

Europe's research after Maastricht

Whenever the European enterprise takes another step forward, its leaders say there will be no change. The European Communities' research policy is just one illustration that such a doctrine does not make sense.

THE time has come round in the European Commission's calendar (see page 5) when another set of committee meetings will set out to define yet another "framework" programme, the commission's name for the programmes of largely applied research which it plans and administers on behalf of the European Communities (EC). There will, no doubt, be the usual trimming of the sums requested (about \$2 billion over four years), but there is unlikely to be root and branch objection of the kind raised by Mrs Margaret Thatcher's British government in its first few years. The truth is that Europe has grown comfortable with the now-familiar collection of projects in fields such as information technology (ESPRIT), broad-band communications (RACE) and semiconductor technology (JESSI). The question is whether Europe should be so content.

The question is all the more pointed now that Europe is within an ace of having its Maastricht Treaty; the British government now seems confident of winning assent from the British Parliament (although something could still go wrong) while Denmark will probably vote YES in its second referendum on the issue on 15 May. When all the paperwork has been done, the European Communities will be renamed the European Union. Otherwise, and immediately, little will change. The most hard-fought provisions of the treaty, those legislating for monetary union by either 1997 or 1999, are fast becoming dead letters as European economies waywardly diverge from the strict requirements on inflation rates and government budget deficits defined by the treaty. Yet Maastricht is more than a symbolic milestone. It will also be a turning point in the way that Europe regards itself.

Ambitions

Although Maastricht is mainly about monetary union (with something about the powers of the commission and the European Parliament) and the more distant prospect of joint working on foreign policy and security, it will also be taken as (and perhaps mistaken for) a sign of Europe's ambitions for itself. There is a danger of internal contradiction. The preamble of the Treaty of Rome emphasizes two goals: an end to intra-European conflict and prosperity not confined to industrial populations (whence the Common Agricultural Policy). In retrospect, that is a conservative agenda. But the rhetoric of the past quarter of a century has been about prosperity founded on technology as advanced as that found anywhere, and about economic power to match that of the United States and Japan. More recently, with the end of the

Cold War, Europe has also nursed the ambition to recruit as members countries in Northern and Central Europe. It is not surprising that there should seem intolerable tension between the goals of the 1950s and those now in the front of people's minds.

Contradictions

Policy on research illustrates that difficulty and is, indeed, central to it. The commission's goal is to encourage industrial organizations, mostly companies, to collaborate on advanced technology. It is a standard condition of its schemes that partners in its projects should come from at least two member states (but the more, the merrier). The commission expects to pay no more than half the costs of agreed projects. One obvious snag is that the insistence on international collaboration, whatever cultural value it may have, is inconsistent with the Single Market (ostensibly a reality since the beginning of this year). Why should the commission believe that advanced mousetrap manufacture, say, should be represented in at least two member states when one manufacturer might supply the whole of Europe with the products and thus could be better placed to export as well? The difficulty, for the commission, is that if it abandoned the present formula, it would face exactly the same problems as national governments, seeking to foster advanced technology but fearful of the temptation of 'backing winners'.

Luckily, there are other courses the commission could follow. There is much the public sector can do to encourage advanced technology. To ensure that there is a sufficient supply of skilled people is the first of them, which requires that attention should be paid to advanced education. (That is why this journal has consistently argued for a redirection of commission funds towards basic research, for the sake of the skill it can in principle provide.) The public sector can also legitimately support advanced technology by providing standards and testing laboratories. (The commission's difficulties in running the old Euratom laboratories may explain its lack of enthusiasm for the infrastructure of industrial innovation.) Above all, the public sector can hope to cultivate a climate in which innovation prospers, intellectually and financially.

The commission would say that these are precisely the goals of its present research programmes; that may be so, but the reality is different. Too often, companies locked into collaborations with rivals regard commission projects as means of making sure that they, not their rivals, are ahead.



Not all the individual projects are well managed, while there is too little support, beneath the commission's ambitious umbrella of applied research, for the multiplication of modest innovations on which the growth of Europe's prosperity is likely most immediately to depend. In an ideal world, the commission (like national governments) would behave more like Japan's Ministry of International Trade and Industry in relation to industrial innovation. Sadly, the commission has not yet the skill and the reputation to pull off such successes.

Moreover, it is unlikely to be in such a position until it has come to grips with the broader contradictions underlying its current operation — its devotion to the Common Agricultural Policy on the one hand and its ambition to recruit the states of Central Europe as members on the other. There is a prior question: can a European Union committed to maintaining an agricultural industry whose size is historically specified also realistically hope to compete with all comers in advanced technology? Nobody doubts that, in principle, Europe is large and sophisticated enough to be a powerhouse of new technology, but the achievement of that goal can only be impeded by the cost of supporting agriculture (which consumes resources and engages people on traditional, not novel, tasks). Europe's laudable ambition also to become a gleaming citadel of environmental protection (for which the European Parliament was calling the other day) may have the same effect. One of the European enterprise's long-standing problems is that of matching its ambitions with reality.

Agriculture, as it happens, has become one of the chief reasons why progress towards a wider Europe will be complicated. As things are, the states of Central Europe are best placed to export farm produce (and steel) to the West. Although the EC has signed association agreements with these countries, it is prevented by the political need to protect its own industries from allowing imports of these commodities on the scale that would be possible. The result is that Central Europe must make do with financial subsidies instead, as if it were an undeveloped region of the world. That is demeaning, uneconomic and a recipe for disaffection. The obvious danger is that if they are unable to earn a living for themselves, the states of Central Europe will slump back into their earlier condition.

Is that what the brave new European Union wishes? If not, the ratification of Maastricht should not be taken as a signal that the Europe of the past few decades has been assured of perpetuity, but rather that a redefinition of its ambitions is overdue. One uncomfortable consequence of the seemingly recognition of the needs of the rest of Europe is that there is no way of bringing Central Europe into the fold except by letting its members earn their keep, implying a repartition of the industrial division of labour now in place. That will mean change not only in the East, but also the West of Europe. The current problems of Germany are an object lesson. The constant repetition by European politicians in the past few months that Maastricht will not necessarily mean change may have been tactically expedient, but squaring the circle in which the EC has snared itself requires, on the contrary, upheaval, not just in research policy. □

Sob tale of the rich

The decline of a London insurance market is a warning and a threatened cost to everybody.

LLOYD'S of London is a unique means by which people anywhere may buy insurance against risks of all kinds, but which has now fallen foul of risks its members should themselves have anticipated. Lloyd's has hitherto relied on the promises of rich men and women to meet exceptional insurance losses from their personal resources; in recompense for this personal risk, they were often handsomely rewarded. But in the past few years, the insurance market has been losing money, and it has turned out that the resources of even the richest people are less than infinite.

Lloyd's lays many of its troubles at US doors. During the past decade, the zealous attempt to rid public buildings in the United States of the flimsiest traces of asbestos, and the personal damage claims that have simultaneously multiplied, have been financed to a substantial degree by the people at Lloyd's. So too have the damages awarded by US courts against manufacturers found to have polluted the environment to a degree that has recently been decreed unacceptable. The implicit complaint is that many British insurers would not have been saddled with these claims if US law and regulation had not been retrospectively changed. There may be something in the view that many environmental regulations in the United States are over-stringent, but an insurance market that boasts of insuring all risks, and which is paid for doing so, cannot logically complain when unforeseen risks are realized. Maybe it has been charging too little for the uncertainties involved, but that is another matter.

The real difficulty now is that risk insurance will be harder to buy, and will certainly be more expensive. And that will put a brake on industrial well-being. For companies need protection from potential catastrophe if they are to remain productive, even in business. Implausible though it may seem, insurance companies also provide a social service.

That is why it is important that the recent troubles at Lloyd's have more to do with its constitution and management than with the riskiness of the world in which the organization operates. For more than a decade, Lloyd's has been riven by scandals of various kinds; professionals employed to assess the riskiness of insured risks and apportion them among the rich risk-takers have been discovered to have salted funds away on their own account. Others have specialized in insuring only the more hair-raising elements of their colleagues' risks, which is a recipe for wealth when times are good and bankruptcy at other times. Yet in 1982, Lloyd's won exemption from the Financial Services Act by which the British government sought to protect ordinary people from financial charlatans, accepting instead that Lloyd's should regulate itself (which it has failed to do). The proposed remedy is that the liability of those carrying the risk in the insurance market should not be unlimited and that capital should be recruited from corporations as well. What that means, sadly for us all, is more expensive insurance. □

International gamma-ray project favoured for ESA launch in 2001

London. A new gamma-ray observatory satellite to be launched in 2001 seems likely to become the first joint space mission between European, Russian and US space agencies. The satellite would cost an estimated £400 million (US\$600 million) and would be between ten and 50 times more powerful than the two gamma-ray observatories now in orbit.

As currently planned — and providing formal approval is obtained from each of the three space agencies — the project, known as INTEGRAL (for International Gamma-Ray Astrophysics Laboratory) would be run by the European Space Agency (ESA) as the second 'medium-sized' project in its science programme, Horizon 2000. One of the two main instruments, a germanium spectrometer, would be provided by the US National Aeronautics and Space Administration (NASA), which has also been asked to make available data-receiving facilities at two ground stations. The satellite would be launched by a Russian Proton rocket from the Baikonur Cosmodrome in Kazakhstan, in return for which scientists from the Russian Space Research Institute (IKI) would have access to the data.

INTEGRAL was given top ranking over three rival projects last week at a meeting in Paris of ESA's space science advisory committee. The choice followed a two-day meeting at the headquarters of the United Nations Educational and Scientific Organization (UNESCO), in which details of the four projects were presented to 250 space scientists.

In addition to the germanium spectrometer, INTEGRAL will carry a caesium iodide imager and two smaller instruments, an optical transient camera and an X-ray monitor. The overall goal is to improve both the angular and spectral resolution of gamma-ray observations. These will be made primarily in the plane of the galaxy, although observations will also be made of gamma-ray sources from outside the galaxy. INTEGRAL will follow up observations from the Russian GRANAT mission (which uses the French gamma-ray telescope SIGMA) launched in 1989 and now coming to the end of its mission, and the US Compton Gamma Ray Observatory, which was launched by the space shuttle in April 1991.

The advisory committee's recommendation now goes to the ESA's science programme committee, representing the scientific communities of its 13 member states, which meets in early June. The committee must also take into account the project's

international and financial implications.

INTEGRAL appears to score highly in all three areas. The international cooperation is seen as a way to reduce costs. The US contribution of \$70 million is likely to come from a NASA programme to support international collaboration, and the overall cost to the European agency is estimated to be within the budget of £260 million.

ESA's medium-sized missions are intended to complement the four larger missions that form the cornerstones of its scientific programme: the SOHO solar observatory, the XMM observatory for X-ray astronomy, a far-infrared space telescope and the cometary probe Rosetta. Thirty proposals were submitted for the 2001 launch, of which four were selected for further study.

Last week's meeting in Paris presented the results of a two-year study of the competing projects. INTEGRAL's main competition in the astronomy field is a project called PRISMA, an all-European mission to study the internal structures of stars by looking at seismic oscillations on stellar surfaces. The two other finalists are MARSNET, a scheme to set up a network of seismological and meteorological stations on the surface of MARS, and STEP, a set of instruments designed to provide

accurate tests of the principle of equivalence between inertial and gravitational mass.

Although all four projects received high scores for their scientific content, PRISMA appears to have suffered from the high costs of its proposed European launch, and MARSNET from uncertainty over US plans for the study of the planet, while STEP was seen by some participants as requiring further technical refinements. Each will still be eligible for submission for the next 'medium-sized' mission to be launched in 2003.

British space officials do not expect any major difficulties in finding the money for INTEGRAL, because ESA science programmes are paid out of the subscriptions of member states which are calculated several years in advance. There is a similar feeling in Bonn that ESA's science programmes provide good value for money, in contrast to high-prestige projects such as the planned spaceplane Hermes and participation in the US space station. The collaboration also coincides with the desire of Germany's new science and technology minister, Matthias Wissmann, to make use of "existing investments" for research.

David Dickson

Study proves Iraq used nerve gas

Washington. An analysis of soil samples from a Kurdish village has proved for the first time that in 1988 Iraqi armed forces used nerve gas as well as mustard gas in air raids against civilians.

Physicians for Human Rights, based in Boston, and the New York group Human Rights Watch brought the samples back last summer and arranged for their analysis at the British chemical weapons laboratory at Porton Down, Wiltshire. The group says that the survival of evidence over a period of years defies expectations and improves the prospects for enforcement of the recently signed international Chemical Weapons Convention (see *Nature* 361, 105; 1993). "This is the first time scientists have been able to prove the use of chemical weapons, and specifically nerve gas, years after the event", says Eric Stover, director of Physicians for Human Rights.

The study found a small number of parts per billion of isopropyl methylphosphonic acid (iPMPA) in soil from bomb craters in the village of Birjinni in

northern Iraq near the Turkish border. Alastair Hay of the University of Leeds, who chairs the group's chemical weapons group, says that iPMPA is a "unique fingerprint" of the nerve gas GB, often known as Sarin. The air attack occurred in August 1988 during a campaign against the Kurdish separatist movement.

Hay says that Sarin would normally degrade in an open environment in a matter of hours. There are no published studies on the survivability of its degradation products, although some classified work may have been carried out.

The human-rights group hopes that the findings will discourage the proliferation of chemical weapons because of fear that their use will be discovered. "The message for those involved in chemical warfare is that they might be detected, even years afterwards", says Hay. However, as many as two dozen countries are still thought to be developing chemical weapons, and the treaty will not come into force until 1995 at the earliest.

Colin Macilwain

Wolfson Foundation's policy on animals angers other British medical charities

London. British medical charities are concerned that a refusal by one foundation to provide grants for experiments involving animals may stimulate the anti-vivisectionist movement and increase the difficulties facing medical researchers who consider such experiments to be essential.

The criticism follows complaints to the Physiological Society by some of its members about guidelines issued to potential grant applicants by the Wolfson Foundation, one of Britain's leading sponsors of university research. These state that the trustees of the foundation "do not normally make grants for . . . research involving animals".

There is particular concern about the impact of this restriction on applicants for the foundation's 'intercolated' awards, which enable medical and dental students to carry out a research project leading to a BSc.

Barbara Rashbass, director of the foundation, says that charitable bodies are entitled to decide how to allocate their funds and that unsuccessful applicants are free to apply for support from other medical research charities, none of which has similar restrictions. Last week, the Physiological Society decided that it would not pursue the matter at the present time, and would accept the foundation's argument.

The Wolfson Foundation spends about £17 million a year on medical research (compared with a total of about £400 million spent by the other charities). The guidelines are widely believed to reflect the personal antipathy towards animal experiments of the foundation's chairman, Lord Wolfson.

The foundation does support research using animal tissue. And it has also provided financial backing for research into alternatives to the use of animals. Thus it has

supported research in the department of human morphology at the University of Nottingham into the possible use of the fluorescein leakage test as an alternative to the Draize rabbit eye irritancy test to measure the potentially harmful effects of cosmetics.

Although there is little criticism of the foundation's decision to support such research, other medical charities argue that its explicit exclusion of research on animals may encourage other smaller research-funding bodies to take a similar stand, a move that could make life increasingly difficult for medical researchers. The larger charities — which have recently formed a group known as Research for Health Charities to respond to criticism of the use of animals in experiments — also feel that Wolfson's stand undermines their attempts to establish a united front.

Glowing report may not avert closure of Dutch primate centre

Munich. Europe's leading primate centre, which pioneered bone-marrow transplantation in the 1960s and which now supplies animals for AIDS research, faces a financial crisis that could lead to closure. Although an independent report on its activities has recommended continued support for the centre at Rijswijk in the Netherlands, many fear that the Dutch government will adhere to an austerity programme that provides no money to operate the centre.

Cuts last year in the Dutch health budget were passed on to the national applied research organization (TNO), whose own grant is being halved by 1994. In response, TNO told the primate centre and its associated radiobiology and immunology research institute ITRI to make good a deficit of around Dfl3.7 million (US\$2 million). The pros-

pect that the primate centre could be closed raised such a storm of protest that the government agreed to delay a decision until after the completion of a report on the centre's performance by the Royal Netherlands Academy of Arts and Sciences.

The international investigating committee, chaired by Jon van Rood, professor of immunohaematology in Leiden, has strongly recommended that the centre and its research facilities should be preserved despite the cost. It judged the centre, which carries out work on AIDS, infectious disease and transplant research, to be a world-class laboratory and says that it offers unique facilities to researchers in Europe such as a rhesus colony for which the microbiological status is known and typed for major histocompatibility complex, essential for transplantation studies and infectious disease research. It also has the only breeding colony in Europe for chimpanzees.

An estimated 5,500 primates were used in Europe last year, according to the report, mostly by the pharmaceutical industry. The primate centre received requests for 300 macaques and 14 chimpanzees, many of which could not be supplied because of a policy that it should be primarily a supplier for TNO institutes.

The report proposes a new structure for the centre, which it says has been underused as a TNO institute. It recommends that it should be taken over by the academy because of its focus on basic research and that it should at the same time establish links with a university. It also recommends that the European Communities (EC) adopt the

centre as a 'Grande Installation' under their Human Capital and Mobility scheme, coordinating its activities with those of the other two European primate breeding and research units, in Göttingen, Germany and Strasbourg, France. The EC has shown interest in the idea, which could provide the centre with Dfl1.8 million per year.

Transferring the centre to the academy, however, will not solve the fundamental problem of tightening government budgets. The academy has already said that it will consider the proposition only if the government provides continuing support to operate and maintain the facility, which the report estimates would require Dfl7 million (US\$4 million) a year. If that is done, according to the report, the centre could attract at least Dfl10 million a year from national and EC grants, industrial contracts and the sale of animals.

But the government is unlikely to want to foot the bill. TNO vice-president Arthur Rörsch says that the government is pleased that the centre is valued so highly but that, "against a background of budget cuts, with health budgets particularly under fire, it is not really easy to be optimistic". Minister of education and science Jo Ritzen has promised an answer this month.

If the government decides against underwriting the venture, then the centre must decide the fate of hundreds of primates. Some of the chimpanzees could be transferred to zoos, but the chimpanzees infected with AIDS must be cared for in special facilities until their deaths.

Alison Abbott



These primates face uncertain futures.

"I am sorry that a well-respected body such as the Wolfson Foundation should have taken this step" says Major General Leslie Busk, director-general of the British Heart Foundation and chairman of the Association of Medical Research Charities. The association, all of whose members are required to sign a statement pledging their support for the use of animals in research, is planning to raise the issue with the Medical Research Council. Wolfson is not a member of the association and, with its current policy, would not be eligible for membership.

Other charity administrators say that Wolfson's policy could help to legitimize the campaigns of anti-vivisectionists and could be seen as implicit criticism of the efforts of laboratory researchers. "If you happen to be using animals, this is like a kick in the teeth", says one. "If all the charities followed the Wolfson's example, medical research would come to a standstill."

Such arguments are dismissed as overreaction by those campaigning for a significant reduction in the use of animals in research. "I would prefer to see the medical charities emphasizing the large amount of research they sponsor into alternatives to the use of animals, rather than making a big fuss out of this", says Michael Balls, chairman of the trustees of the Fund for the Replacement of Animals in Medical Experiments, and until recently professor of medical cell biology at the University of Nottingham.

Further support comes from Sir David Phillips, chairman of the Advisory Board for the Research Councils and a trustee of the foundation. "Wolfson's policy could be seen as an inducement to people to devise experiments which do not involve the use of animals, and almost everyone would agree that that is a good idea", he says.

David Dickson

Research ministers approve Framework

Munich. The council of research ministers of the European Communities (EC) last week approved the structure and the proposed ECU13.1 billion (US\$15.8 billion) budget for the EC's fourth framework programme, which will fund research from 1994 to 1998. The positive response raises hope that the final text of the programme could be approved by the commission, council and parliament by the end of the year (see *Nature* 362, 778; 1993).

EC research commissioner Antonio Ruberti also announced preliminary plans to improve programme management by simplifying and decentralizing procedures. Details are not yet available, but one proposal is to establish regular deadlines for grant applications and to require for the first selection round a short summary of project proposals rather than the full 20-page application, as is now the case.

A.A.

Japan adds supercomputers in one-time boost to budget

Tokyo. Research institutes of the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) will receive supercomputers in a supplementary budget designed to help pull Japan out of recession (see *Nature* 362, 381; 1993). Many Japanese national universities will also benefit by getting much-needed Local Area Networks (LANs) to link on-campus computers. But an attempt by the science-related ministries and agencies and the ruling Liberal Democratic Party (LDP) to create a new budget category for these items and to crack the rigid ceiling on outlays for science has been defeated by the Ministry of Finance.

The supplementary budget, details of which are expected to be approved by the cabinet in the next few weeks, will include ¥25–30 billion (US\$225–\$270 million) for 11 supercomputers and mini-supercomputers to be shared among six science-related ministries and agencies (see table). This is three or four times the number normally purchased each year by the government.

MITI will receive three, including one for its new National Institute of Advanced Interdisciplinary Research in Tsukuba which will be the centre for a large-scale government-industry project on nanotechnology. STA also gets three and the Ministry of Education, Science and Culture gets two for national universities or university-related institutes.

The computer for the National Cancer Center will form the central database for an online national network of imaging data on cancer patients. The supplementary budget is also said to include several billion yen to set up the network, part of the next ten-year cancer research programme to begin next year (see *Nature* 361, 672; 1993).

The Ministry of Agriculture, Forestry and Fisheries is expected to use its supercomputer for the rice genome project at the National Institute of Agrobiological Resources in Tsukuba, but the ministry has yet to reveal its plans. And the Ministry of Posts and Telecommunications will get a supercomputer for its single institute.

Supercomputers are not the only scientific elements in the supplementary budget. About ¥8 billion will be set aside for on-

campus LANs for 20–30 national universities, according to a government official involved in the negotiations with the Ministry of Finance. A few tens of billions of yen will also be spent on new buildings and facilities for the science-related ministries and agencies.

Where the supercomputers will go

Ministry/Agency	Number	Recipient
MITI	3	Tsukuba supercomputer centre (1); National Institute for Advanced Interdisciplinary Research (2)
STA	3	RIKEN (1) Power Reactor and Nuclear Fuel Development Corporation (1); National Aerospace Laboratory (1)
MESC	2	MESC organizations
MHW	1	National Cancer Center
MAFF	1	MAFF institute in Tsukuba
MPT	1	Communications Research Laboratory

MITI: Ministry of International Trade and Industry, **STA:** Science and Technology Agency, **MESC:** Ministry of Education, Science and Culture, **MHW:** Ministry of Health and Welfare, **MAFF:** Ministry of Agriculture, Forestry and Fisheries, **MPT:** Ministry of Posts and Telecommunications

But the science-related ministries and agencies failed to classify such items as 'new social infrastructure' outside the present budgetary system. Despite the backing of the LDP, in particular Hiroshi Mitsuzuka, chairman of the ruling party's policy research council, the Ministry of Finance argued successfully that such changes require rewriting Japan's finance laws.

The LDP and science-related ministries and agencies are now trying to ensure that next year's budget includes provision for items such as a high-capacity national backbone network for linking computers in government institutes and universities. The ruling party is considering introducing a bill to allow national construction bonds to be used. Alternatively, it might use funds from the sale of government-held shares of Nippon Telegraph and Telephone, Japan Tobacco and JR railways as well as earnings from a proposed system of betting on football matches.

Although government officials are confident of somehow finding a way next year to pay for the national backbone network, they are sceptical of the LDP's ability to break through the ceiling on overall spending on science. The party's most likely recourse is a face-saving compromise with the Ministry of Finance that does not alter the status quo.

David Swinbanks

Stolen report alleging discrimination and favouritism in NIH office raises furore

Washington. An independent report supporting allegations that senior managers in the procurement office at the US National Institutes of Health (NIH) have systematically discriminated against African-Americans and have operated a 'social network' in which sexual favours are exchanged for career advancement has become the focus of a campaign by two civil rights organizations to improve conditions for blacks at NIH. Their anger has been fuelled by the theft of the report from the consultant's office on the morning after it was submitted to NIH last autumn — including the codes intended to provide anonymity for witnesses who supplied the consultant with evidence of misconduct.

The report was prepared by Benjamin Alexander, a former senior US government official and a former university president who was trained as a chemist. Alexander was hired to investigate the validity of complaints of discrimination and favouritism dating from 1987 against the office of acquisition management within the NIH director's office. In the course of the five-month study, Alexander interviewed several dozen witnesses who, under the promise of anonymity, described an "ole boy network" of preferential hiring and promotion, a pattern of unfair performance ratings and the use of inappropriate educational requirements for available jobs.

Those conclusions were reached on the basis of answers from a control group of employees in the office that matched the administrative levels of those accused of acting improperly and the racial composition of those making the allegations. Alexander found that the control group shared the views of the complainants and disagreed with those supporting the managers in 18 out of 20 areas.

On 19 September, the day after Alexander submitted the report to Diane Armstrong, director of NIH's Office of Equal Opportunity, thieves broke into the offices of Alexander's company, Drew Dawn & Associates of Silver Spring, Maryland, and stole the report along with the codes. The local police found no incriminating evidence at the scene and they believe that the theft, which did not involve office equipment and other items of value, was done by professionals.

Last week, the Montgomery County (Maryland) chapter of the National Association for the Advancement of Colored People (NAACP), tired of waiting for the police to solve the case and for NIH to comment on the report, held a press conference to express its outrage at the events of the past seven months and to demand that the lives of those affected "be made whole". The group, along

with the NIH chapter of Blacks in Government (BIG), wants the Federal Bureau of Investigation to help solve the break-in and wants NIH to act promptly to resolve the complaints of discrimination and to punish those responsible.

The civil-rights groups are particularly upset with NIH's response to the news that the confidentiality of those who provided information to Alexander had been breached. Within 24 hours, NIH security police were given the list of names and told to call each person to tell them of the theft. NIH officials say only that the witnesses were notified "in the most expeditious way possible"; members of BIG insist that a telephone tree was created in which one person was told to call several others. In any case, the names of those who had provided confidential information were soon common knowledge within the relevant offices at NIH.

NIH says that criticism of its response to both the report and the theft is unfair. Armstrong says that the report has convinced NIH officials that "there are some

serious problems in the office of acquisitions management" and that, as a result, NIH has hired another consultant "to advise us if there is information that we can act upon". The Alexander report was revised four times before it was acceptable, according to Armstrong, who said that the final version "does not contain any sworn statements or evidence that could provide the basis for specific actions against anyone". In the interim, she adds, NIH has scheduled training on sexual discrimination and cultural awareness for the entire division. As for the break-in, Armstrong says that the report "was not our property" at the time it was stolen, so it is not clear what role NIH should play in an investigation.

The civil rights groups say that NIH is not doing enough. "We're not happy with what is going on", says Vincent Thomas, president of the BIG chapter at NIH. "We've said all along that the key is managerial accountability, and until NIH is willing to punish people for their behavior, nothing is going to change." **Jeffrey Mervis**

Russia gets first batch of emergency grants

Moscow. With 100 \$500 grants already on their way to scientists working in Russia, the International Science Foundation (ISF) has taken the first step towards meeting its goal of helping to preserve research in the former Soviet Union.

The foundation, created last autumn by US financier George Soros, expects to make a second set of emergency grants to as many as 20,000 scientists after the 31 May deadline for applications. ISF officials are selecting researchers on the basis of the quality and quantity of their published output (see *Nature* 362, 95; 1993), although only a portion of the 35,000 who are eligible are applying for money to keep their laboratories going. Scientists may also be reimbursed for the cost of attending international conferences.

Segments of the Russian press have criticized the ISF and point to the modest size of ISF grants as evidence that the foundation is trying to 'buy' Russian scientific brainpower at a fraction of its real value. Others are grateful for the additional money but believe that the ISF should work more closely with government agencies. Aurab Yakobashvili, deputy minister of science, says that the lack of such coordination makes it more difficult for the government to plan for the future of Russian science. Still others have raised questions about the criteria adopted by the ISF for judging applicants.

Alex Goldfarb, executive director of the

ISF and formerly a biochemist at the Engelhardt Institute in Moscow, sees the gap between the number of applicants and the number of eligible scientists as a sign that many of his colleagues have left the country for greener pastures in the West or have taken up another occupation. The imminent loss of perhaps a third of the cream of Russian science, he says, demonstrates the importance of taking rapid action, by whatever means available, to help those still willing and able to do science.

In addition to the emergency grants to individuals and small teams, the foundation has also given \$500,000 to the Komarov Botanical Institute in St Petersburg for the preservation of one of the world's largest herbaria. Some \$80,000 was awarded to a popular science magazine *Chemistry and Life* and \$4,000 was given to a special high school in Moscow that maintains high standards for teaching physics and mathematics. Soros has promised to spend a total of \$100 million on strengthening science in the former Soviet Union.

Boris Saltykov, the minister of science, believes that Soros's commitment has spurred Western governments into doing more than they would have done otherwise. "I can't prove it", he says, "but I believe that the activities of George Soros helped to push the Clinton administration into taking effective steps to assist Russian science".

Vladimir Pokrovsky

Clinton's plan for 'green car' faces bumpy research road

Washington. The Clinton administration is likely to have considerable difficulty in carrying out a proposed joint 'clean car' research and development programme with the major US car manufacturers.

Diminishing environmental returns from refinements to petrol-driven cars and uncertainty over which alternative power sources to pursue will complicate the plan, according to motor industry experts who last week testified before the clean air and nuclear regulation subcommittee of the Senate Environment and Public Works Committee. In addition, the industry's preference for an inexpensive way to meet tougher environmental regulations leads to different research goals from those of the administration.

There is also concern that too much collaboration between US car makers could blunt their ability to compete in global markets. "If the companies all draw from the same research and technology base, we may inhibit technical and product innovation, just when it is most needed", Albert Sobey, an industry consultant from Michigan, told the subcommittee.

In February, US President Bill Clinton announced his interest in working with the automotive industry on 'clean car' technology. Since then, senior representatives of General Motors, Ford and Chrysler have met several times administration officials led by the president's science adviser, John Gibbons, and Deputy Secretary of Commerce-designate John Rollwagen.

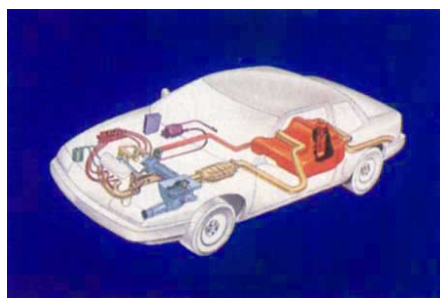
But whereas the administration likes to talk about a greener and more internationally competitive car industry, Detroit wants to spend any available support on overcoming bread-and-butter engineering obstacles in complying with environmental legislation. Laws already passed in California and under consideration in other states would require car makers to reduce each year the hydrocarbon, carbon monoxide and nitrogen oxide emissions of their fleets.

The manufacturers would like to channel any new government money through their existing collaborative research clearing house, the US Council for Automotive Research (USCAR), which coordinates joint research in ten areas, ranging from battery technology to passenger safety. But only one, the Advanced Battery Consortium, has so far attracted significant federal funding, with half of its four-year, \$260-million programme being financed by the Department of Energy. Detroit research managers see the other USCAR consortia as obvious avenues for additional federal support.

Joseph Colucci, an executive director of

research at General Motors, says that a consortium founded last summer on low-emissions technology offers the best match between the administration's goals, the capabilities of the federal laboratories and the industry's priorities. "It would be very supportive to have the federal laboratories working on this issue", he says.

The low-emissions consortium deals with technology that will cut petrol engine emis-



Federal support could help Detroit to develop cleaner emission-control systems.

sions to meet the tightening regulations. Existing catalytic converters can produce extremely low hydrocarbon and carbon monoxide outputs only in optimal conditions. The challenge is to develop converters that will do this at once from a cold start and for the life of the vehicle.

By focusing on this and other problems that USCAR believes are well-suited to collaborative research, the administration would avoid disrupting current plans by Detroit. But it would also miss the chance to coordinate efforts in the most strategically significant areas — such as engine technology — which the 'Big Three' have purposely excluded from USCAR.

Gibbons believes that the Department of Energy weapons laboratories can play a significant role in strengthening the US car industry. But in some areas of apparent synergy, such as materials technology, the motor industry's requirements for cheap mass production differ from the military's emphasis on small-volume, high-quality manufacturing in which cost is secondary.

In the meantime, Detroit hopes to get some help in tackling the research problems it faces in dealing with environmental regulation and is counting on better relations with Washington. "In the past, the industry and the government have had an adversarial relationship in this area", says Colucci. "That should change, but it is only what the Japanese have been successfully doing for years."

Colin Macilwain

Research councils want more control of European projects

Strasbourg. A "club of national research councils, researchers and institutes" to serve European research interests being neglected by the European Communities (EC) has been recommended by participants at a meeting organized last month by France's basic research agency, the CNRS (Centre National de la Recherche Scientifique). They would also like to have an independent body to evaluate European science and special one-year grants to help reintegrate post-doctoral students returning to their country of origin after working elsewhere in Europe.

The recommendations, which reflect concern that the EC is accruing too much power over research, are being presented to France's new research minister, François Fillon. Fillon has pledged to support efforts, particularly those by the CNRS, to increase the control of individual countries over international research collaborations. Widely known for his anti-Maastricht politics, Fillon says that he wants to avoid "getting bogged down at the EC level...whose cumbersome procedures put a damper on the research community".

The proposals are also being presented to national research councils, which have recently discovered that a growing proportion of Europe's research budgets come from the EC's research commission. Meeting for the first time in Bonn in January (see *Nature* 361, 576; 1993), the councils are trying to make EC-supported international research programmes more efficient and have scheduled a meeting in London in October to discuss the issue.

CNRS was host for the meeting partly because of its desire to take a leading role in Europe's changing research landscape. The agency is trying to decentralize French research by moving half of its laboratories to the regions (see *Nature* 356, 373; 1992), and taking a bigger role in international research politics would help to fill the vacuum in Paris.

The CNRS has been less successful in setting up an international company called Euroresearch. Under discussion for two years, the company is supposed to allow France, Germany and Spain to administer EC grants to their research institutes and postdoctoral students.

One problem it would address is the proportion of grants — as much as 10 per cent — that are lost to currency conversions because French research institutes cannot hold foreign currencies. But the plan has foundered on the EC's demand for a general liability clause, a feature that has proved difficult to sort out on an international level.

Alison Abbott

International facilities said to boost national economy

London. A report published by the UK Office of Science and Technology (OST) challenges the traditional view of the Treasury that hosting an international scientific facility has little long-term impact on the economy of the host nation.

The report, prepared by the consulting group Segal Quince Wickstead of Cambridge, says that international facilities can boost the long-term economic fortunes of companies that supply them with equipment and services, act as a magnet for other high-technology companies and generally raise the public profile of science. Although it is difficult to calculate the extent to which the funds represent a net gain to the economy, according to the report, the income and value added tax paid to the government by foreign scientists and other employees represents a clear economic gain.

The Treasury has accepted the idea that international facilities are a cost-effective way to support large-scale research, but it has been reluctant to provide financial incentives to attract such facilities to Britain, following the example of France and Switzerland, because of doubts that they contribute to the country's economic wealth. This conclusion is challenged by an analysis of spending on equipment and salaries at four international scientific centres: the Joint European Torus (JET) at Culham, England; the European Laboratory for Particle Physics (CERN) at Geneva, Switzerland; and the European Synchrotron Radiation Facility (ESRF) and the Institut Max von Laue-Paul Langevin (ILL), both in Grenoble, France.

Overall, the analysis found that between 40 and 70 per cent of the money to operate

the facilities was spent in the host nation. While admitting that some of this funding could have displaced other funding sources — for example, technicians in short supply would likely have found alternative work — the authors of the report, Robin Brighton and Virginia Aschas, say that they also found clear evidence of economic gain.

For example, in many cases local companies providing scientific equipment and other services were able to benefit from reduced delivery costs, personal contacts and a more detailed knowledge of a facility's needs. Those factors give them a competitive advantage in bidding on contracts. For scientists, the main economic advantage of having a facility in their home country is the reduced cost of travel and accommodation.

Brighton says that it is impossible to quantify the likely gains from an international facility, because a number of important factors (such as displacement costs) depend heavily on prevailing economic conditions. He says, however, that the study "produced enough evidence to suggest that the potential economic benefits [from an international facility] should be studied very carefully".

OST officials hope that demonstrating these likely gains will make it easier to obtain Treasury backing for any future bids to locate scientific facilities in Britain. At the top of the list is a European Neutron Source, a successor to the Nuclear Structure Facility at Daresbury that was closed down last month, which many physicists would like to see built at the Science and Engineering Research Council's Rutherford Appleton Laboratory.

David Dickson

Swiss look for ways to reap benefits of next Framework

Basel. Swiss researchers, who lost access to millions of dollars from the European Communities (EC) when voters decided in December to delay the process of joining the EC, are waiting for the reaction to their government's attempt to participate at some level in the EC's fourth Framework Programme, which begins next year.

The referendum blocked Switzerland's entrance into the European Economic Area (EEA), the first step towards full EC membership. Switzerland already participates in some 200 individual EC projects, paying researchers the costs normally paid to full EC members, which average SFr30 million (US\$20 million) a year.

The change in status would have allowed Switzerland to participate fully in broader Framework programmes, although it would not yet have been eligible for direct financial support. Swiss researchers would also have been allowed to join programme committees and to serve as project leaders on individual projects.

The vote deprived Max Hess, an immunopathology professor at the University of Berne, of a promised position on the Biomed programme advisory commission. A third of the 140 projects in this programme have Swiss participation, he says. "The worst thing is that we get no information about what is going on", he says.

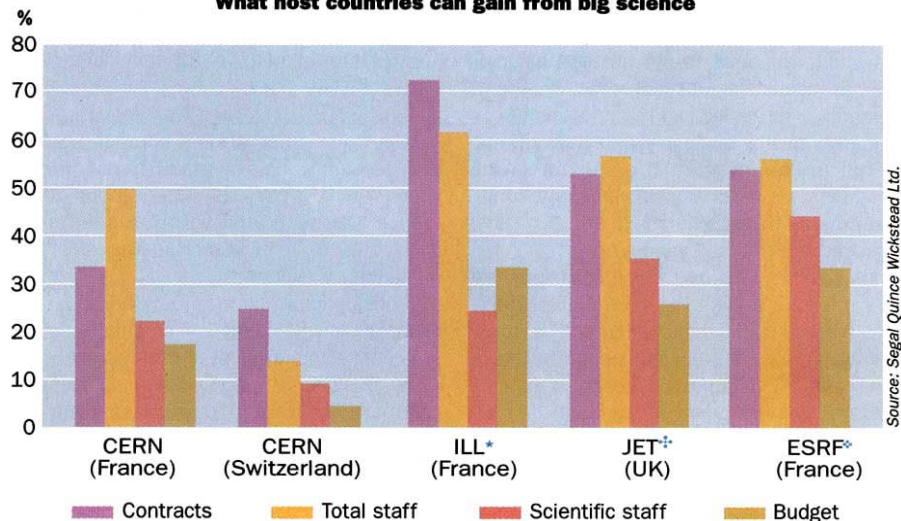
Fred Paccaud, a professor of social medicine from the University of Lausanne, held a special exemption to serve as a project leader in the MR4 programme, a predecessor of the Biomed programme, but his proposal to continue working on Biomed was rejected in January. "I was told I am no longer entitled to apply", he says.

Tim Guldemann, head of economics and foreign policy in the Swiss science policy coordinating group, says that all sides suffer from the exclusion of Swiss researchers. With three per cent of its gross national product devoted to research and development, the country ranks with Japan as the heaviest investor of science in the world.

The Swiss government was prepared to pay SFr477 million to participate in the fourth Framework programme and had encouraged researchers and industry to submit proposals. These proposals are now being used as a negotiating tool to demonstrate Switzerland's strength in particular areas. The EC Council of Ministers needs to establish a general mechanism for negotiating with Switzerland now that it is the only country in Europe that has neither EC nor EEA status. Last week it agreed in principle that Switzerland could take part in projects where its participation would strengthen the research effort.

Oliver Klaffke

What host countries can gain from big science



* Institut Laue-Langevin, + Joint European Torus, * European Synchrotron Radiation Facility

Source: Segal Quince Wickstead Ltd.

Germany revamps system to fund medical research

Munich. German research minister Matthias Wissmann last week announced a major restructuring of medical research funding that for the first time establishes independent external refereeing for projects in universities and their associated clinics.

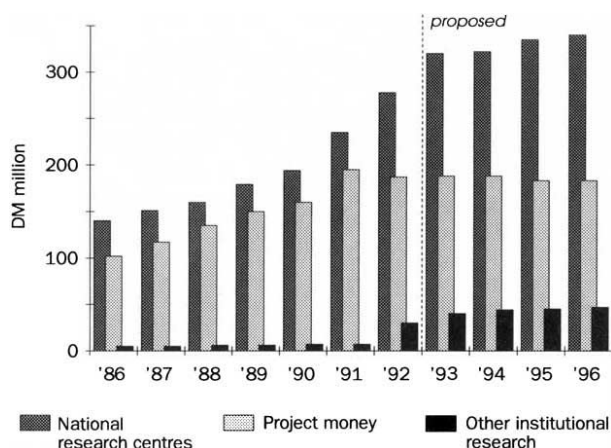
tend to be small and isolated, shunning collaborative links with other small groups, either locally or nationally.

Wissmann wants to change this. The BMFT is allocating DM250 million over the next eight years to support six to eight

interdisciplinary research centres. These centres will be composed of several research teams, usually local, that focus on defined collaborative projects. The projects undertaken by the centres will be assessed by a formal refereeing systems of international standards.

Most of the cost will at first be picked up at the federal level, but the BMFT will gradually reduce its share of financial support over the eight years. The clinics will have to make up the difference with funds from the *Länder*, thereby ensuring that the money is used for

A new plan for German health research



Wissmann hopes that his plan, called 'Health Research 2000', will eliminate inefficiencies within German biological research (see *Nature* 357, 182; 1992) and strengthen the weak links between basic medical and clinical research.

Some DM2.15 billion (US\$1.36 billion) has been set aside by the research ministry, the BMFT, to support the first three years of the plan. Around DM1.4 billion will be used to fund medical research in the large national research centres, such as the German Cancer Research Centre in Heidelberg, and DM750 million will be spent on individual projects in other research institutes or universities. The BMFT hopes that new methods for allocating money will encourage a restructuring of university research which, it believes, is in urgent need of reform.

Although university researchers can apply for BMFT project grants, universities receive their basic research money from their local (*Länder*) governments. This money is not usually distributed on a truly competitive basis, a process that is widely criticized.

Clinical research is carried out mostly at university clinics, which receive 80 per cent of their cash from medical insurance companies and the rest from the *Länder*. Although this 20 per cent is intended for teaching and research, much of it is used to make up deficits in general health-care budgets. Inefficiency in the clinics and the universities is compounded by the fact that research groups

its intended purpose.

Remaining funds will be allocated to individual projects, a third to public health research and two-thirds to clinical research. Joint applications will be shown preference, as well as projects that "strengthen cooperation between basic and applied [clinical] research".

Alison Abbott

Montagnier to assess French AIDS effort

Paris. Luc Montagnier of the Pasteur Institute in Paris has been appointed to lead a panel reviewing France's effort against AIDS. The breadth of Montagnier's assignment — to examine social and public health issues as well as current research — has prompted speculation that the government may appoint him to a new position of national AIDS coordinator.

"The government considers that more needs to be done", says Montagnier, whose preliminary report is due in August. Although he says that he intends to keep an open mind, Montagnier has criticized the government in the past for not supporting more research outside the mainstream and for not paying more attention to Africa. In January, he and Federico Mayor, director general of UNESCO, created the World Foundation for Research and Prevention of AIDS (see *Nature* 361, 102; 1993). **D.B.**

Whaling meeting expected to leave issue unresolved

Tokyo. Despite years of debate and scientific analysis, next week's meeting of the International Whaling Commission (IWC) is unlikely to resolve the fate of commercial whaling.

Anti-whaling forces at the annual meeting in Kyoto do not have sufficient support for a French proposal to create a whale sanctuary in the southern ocean which would prevent the Japanese from resuming commercial whaling. Neither does Japan have the votes to achieve a resumption in commercial whaling, despite evidence from the IWC's scientific committee that there are sufficient numbers of minke whales.

In 1982, the IWC introduced a moratorium on commercial whaling on the basis of incomplete data on the size of the population and whether it was sufficient to sustain commercial activity without risk to the species. By 1991, the IWC scientific committee had developed a revised management procedure (RMP) to provide adequate safeguards and had estimated the population of Antarctic minke whale at 760,000, well above the number needed to resume commercial whaling (see *Nature* 357, 532; 1992). But the IWC has yet to decide whether to continue or end the moratorium, preferring to ask more questions of the scientific committee regarding the management procedure (see *Nature* 358, 99; 1992).

"My guess is the commissioners will be desperate for a reason to postpone a decision", says one scientist participating in the closed scientific committee meetings that have preceded next week's plenary session. "If they voted to lift the moratorium, they'd get lynched at home."

A proposal by France to create a whale sanctuary in the southern ocean below 40° S may provide grounds for delay. The Japanese are confident they can muster the necessary 25 per cent minority to block the French proposal if a vote is called. Instead, those against whaling are likely to push for further discussion of the proposal.

The Japanese are in no hurry to push for resumption of commercial whaling. "Even if the IWC permits us to resume, commercial whaling [in the Antarctic] has died", says Japan's IWC representative, Fukuzo Nagasaki. "It would take two or three years to develop a concrete plan."

Japan sees its fight to preserve commercial whaling as a test case for its exploitation of other commercially more important marine resources, for example the bluefin tuna in the Atlantic. A defeat in a situation where sustainable management of the resource seems possible could threaten other resources where there is greater cause for concern about depletion.

David Swinbanks

Problems in Ireland . . .

SIR — In your leading article "What Brussels should do for research" (*Nature* 362, 93; 1993) you emphasized the need to increase the quality, quantity and participation in science research in countries such as Ireland. Your article is indeed timely as it is likely that financial support by the Irish government for research will be significantly reduced from its already low level.

I believe that scientists would warmly welcome the development of new strategies that would address many of the problems that are in danger of causing major damage to the scientific base in Ireland. This had begun to develop significantly in the past few years in terms of quality. In addition, a number of new universities and tertiary institutes were established. However, if these and the well-established universities and institutes are to continue and improve, what is required is an increase in support, not a major reduction.

Ireland has a well developed educational system that supplies good candidates for science and technology courses. This is in no small measure due to the out-of-school science activities of organizations such as the Royal Dublin Society and the Aer Lingus Young Scientists Exhibition and many dedicated teachers. Hence, Irish Young Scientists have done remarkably well at international science competitions. At tertiary level, there is a variety of courses of a very high standard. However, the support for research, particularly basic research, is severely limited. This has implications for advanced training of students in techniques relevant to modern industrial processes, it severely limits the ability of lecturing staff to be fully up to date with new technologies and it limits the ability to provide 'state-of-the-art' technology and training at tertiary level for both indigenous and multinational companies. The argument is often made that what is required is applied research to deliver products and processes directly to industry. Undoubtedly, this approach is absolutely necessary but because technology advances and changes so rapidly, failure to keep up will give a very limited life to any industry. There are many examples where what was 'academic' a few years ago is now very much applied. What is required is a balance where basic and applied research co-exist and catalyse further developments.

It is also important to remember that where jobs are indeed a problem, as in Ireland, postgraduate research positions should be clearly seen as making a major contribution to employment — where else would employment cost so little and the employee undergo intensive train-

ing? It is very good value for money.

What Brussels should do is to provide direct support for research students and equipment in the less advantaged areas provided they interact with major European collaborators. In this way all involved would benefit. Otherwise, there is a real danger of a one-way traffic whereby aspiring scientists will move to the well-developed centres and then will be unable to return to improve their countries of origin, due to the lack of positions and of research support. If this happens, surely the whole concept of a truly equal and united Europe will be merely hearsay.

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. . . and Spain

SIR — In addition to the problems encountered by Spanish researchers in finding a post in Spain after they have obtained a doctorate in another country (*Nature* 306, 502; 1992 & 361, 578; 1993), there is also the slowness of the procedure for obtaining recognition of their title. Although this document is an essential prerequisite for the competition for posts in universities and in the CSIC (Spanish Council for Scientific Research), it can be obtained only after a bureaucratic process that takes at least one year and sometimes more than two. During this period, researchers who have obtained their degrees abroad cannot be candidates for these posts and are therefore at an enormous disadvantage in relation to homegrown researchers.

Having applied in June 1991 for recognition of my doctorate, which I obtained in Paris, I presented my candidacy for a CSIC post as a palaeobotanist. I was accepted as a candidate — indeed, the only candidate — for this post, but in March 1992 the jury refused to let me take the examination owing to the absence of the document recognizing my title. Despite the decision of the president of the CSIC to readmit my candidacy, the jury, in June 1992, refused to examine me once again. In July I was readmitted a second time, having finally obtained recognition of my French doctorate after more than a year. Despite this, the new president of the CSIC, José Mato, decided in November to exclude me from the examination and to cancel the competition. As I was the only candidate, the post has been lost.

The opposition displayed by certain Spanish scientists towards the return to Spain of young researchers trained

abroad often takes advantage — with the complicity of the administration — of protectionist bureaucratic mechanisms dating from the Franco period. The time wasted in awaiting recognition of their title puts a substantial block on these young researchers at the most critical point in their career.

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. . . and Italy

SIR — It has already been reported that, contrary to official policy, the scientific merits of candidates for academic promotion in Italy are not given primary consideration by the members of the judging commission, so it often happens that a loser has a *curriculum vitae* (c.v.) clearly superior to that of a winner¹. Unfortunately, it may also be superior not only to those of the winners but also to those of the commission. A recent experience of mine is a case in point.

I participated in a national competition called to fill eight positions of associate professor in gastroenterology². A seven-member commission had the job of ranking the candidates on the basis of their scientific qualifications and on their performance at an oral examination consisting of a discussion of their scientific work and an academic lesson. Before the examination, one of the commissioners told me that I would not be one of the winners because the positions had already been assigned to others who were, for the most part, assistants to the various commission members. As forewarned, I did not win, despite a good oral presentation and the best c.v. of all the candidates. One of the most disturbing aspects of this experience, which I think merits emphasis, is that my c.v., rated on the basis of the number of publications (Medline Data Base), on the number of citations in the literature (*Science Citation Index*), and the impact factor¹, was not only better than that of any of the winners but also of those of the commission members as well. By bringing still more evidence to light, I hope I have made some contribution towards reforming an outmoded and unfair system, one that permits arrogant and unscrupulous commissioners to do as they please.

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1. Gaetani, G. F. & Ferraris, A. M. *Nature* 353, 10 (1991).
2. *Gazzetta Ufficiale della Repubblica Italiana* n. 70-bis, 4 settembre 1990, e n. 47-bis, 14 giugno 1991.

Progress with inventories

SIR — When regretting the lack of an inventory of life (see Gaston and Mound *Nature* **361**, 579; 1993), we should be careful to distinguish the complete list of organisms, that is including many as yet undiscovered species, from the smaller set of just those that have already been described. That we have no inventory of the former is inevitable (we have neither seen them all, nor estimated with certainty how many millions there are), whereas the more surprising omission is that there is no master inventory even of those species that have been dealt with by taxonomists.

Gaston and Mound offer sensible advice: concentrate our forces on selected groups. What they omit to mention is that substantial progress is now being made in doing exactly this, the production of master inventories for selected groups. Be it of bacteria^{1,2}, protists³, insects⁴, molluscs⁵, fungi⁶, plants^{7,8} or plant fossils⁹, a series of global synonymized taxon checklists are making progress. These are databases carefully prepared by teams of specialists to be available soon on-line or distributed on disc. Producing master catalogues requires specialist software (for example, ALICE¹⁰, "Linnaeus"¹¹, pcTROPICOS¹²) and cooperative management to obtain a very wide range of taxonomic expertise. New levels of international organization have been needed in projects such as the ILDIS Leguminosae database⁷ to turn regional monographs into workable worldwide classifications and to select a preferred reference system where taxonomists debate alternative taxonomies.

The first public taxonomic databases have experimented with the handling of alternative taxonomies (as in the US Nature Conservancy's taxonomic inventory with local variants), with attaching biological data in such a way that it can be refreshed for subsequent taxonomic changes (for example, ILDIS/Chapman and Hall Leguminosae phytochemical database¹³), and with the use of images (such as *The Plant Fossil Record*⁹). The adoption of standards by TDWG¹⁴ networking and the first steps towards 'taxonomically intelligent' integrity checking are enabling a second generation to appear. Gaston and Mound may have to wait before the 750,000 insects can be handled, but IOPI¹⁵ is already building on the expertise of ILDIS⁷, TROPICOS¹⁶ and the Australian Plant Census¹⁷ to create a system for the

world's 250,000 vascular plants. ETI³ is similarly working on protists, birds and molluscs.

If insects are being named at the rate of about 7,250 species a year and synonymized at about 1,450 a year, then these rates are within our capacity for entry into master inventories. Resources are, however, a major limiting factor. It remains something of a surprise that at a time when conserving species diversity is valued so highly, so little priority is given to listing the basics of what species there are to be conserved or lost. But substantial progress is being made and the most important resources of all, expertise and know-how, are now becoming available. Do not despair!

Frank A. Bisby

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1. DSM. *List of Valid Bacterial Names* (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig).
2. BIOSIS TRF. *BIOSIS Bacterial Taxonomic Reference File* (BIOSIS, Philadelphia).
3. ETI. *Linnaeus Protist* (also Mollusc and Bird systems) (Expert-Center for Taxonomic Identification, Amsterdam).
4. ANI. *Arthropod Name Index*. (CAB International, Wallingford).
5. Eschmeyer, W.N. *Taxonomic Database for Fishes* (California Academy of Sciences, San Francisco).
6. IMI. *Species Fungorum Database* (International Mycology Institute, Egham).
7. ILDIS. *International Legume Database & Information Service* (ILDIS Phase 1, Version 1.8) (ILDIS Co-ordinating Centre, Southampton).
8. CITES. *CITES Cactaceae Checklist* (Royal Botanic Gardens, Kew).
9. IOPB. *The Plant Fossil Record Database Version 1.0* (International Organisation of Palaeo-Botany, London).
10. ALICE. *Species Diversity Data Management System* (ALICE Software Partnership, Kew).
11. Linnaeus. *Multi-Media Taxonomic Information System* (ETI - Center, Amsterdam).
12. pcTROPICOS. *Comprehensive Database Management System for Systematic Botany*. (Missouri Botanical Garden, St Louis).
13. ILDIS/Chapman & Hall. *Legume Phytochemical Database* (ILDIS Phase 2, Phytochem. Module, Version 0.7). (ILDIS Co-ordinating Centre, Southampton).
14. TDWG. *International Working Group on Taxonomic Databases for Plant Sciences* (a commission of the International Union of Biological Sciences).
15. IOPI. *International Organization for Plant Information*.
16. TROPICOS. *The Botanical Database of the Missouri Botanical Garden* (Missouri Botanical Garden, St Louis).
17. Hnatiuk, R.J. *Census of Australian Vascular Plants* (Bureau of Flora & Fauna, Canberra, 1990).

SIR — Gaston and Mound consider that "for the foreseeable future, description of all the world's species will remain impossible". They are right. It is impossible to describe all the species that currently inhabit the planet given, for example, that species evolve and that there would be no obvious way of knowing when the table was complete.

The more practical challenge that faces the taxonomic community is to describe and classify all the species that are known to inhabit the world. This task is not impossible, just very difficult.

The productivity and size of the work force involved and the rate at which undescribed species become known are clearly important factors.

The Instituto Nacional de Biodiversidad (INBio) in Costa Rica has shown that, together, parataxonomists and taxonomists can make astonishing progress towards an inventory of species. INBio provides a model that other tropical countries are already starting to follow.

Information technology lies at the heart of almost all current projects and international initiatives such as the International Organisation for Plant Information (IOPI) are converging on common approaches. The taxonomic literature and major catalogues of species are clear targets for conversion to machine readable form and technologies for automating this process are increasingly being used.

Gaston and Mound are right that in taxonomy, as in science in general, the drive comes from individuals. But the community comprising these individuals is responding to the biodiversity crisis by setting priorities and defining focused research programmes. In the major taxonomic institutions, the emphasis is firmly on collaboration and concerted action, as initiatives such as the recently formed European Museums Network emphasize.

New ways of working will take taxonomy into the fast lane, providing information on species, the components of biodiversity. This is essential if the scientific quality of studies of biodiversity at the ecosystem and genetic level is not to be compromised.

Stephen Blackmore
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SIR — I believe that if a current inventory of known insect and other species was available, descriptive work might be more efficient than the current 20 per cent wastage rate (due to synonymy).

But we are faced with a cultural problem where descriptive work goes unrewarded, and systematics and taxonomy are often treated as one. With encouragement, a competent amateur can diagnose species and varieties without resort to parsimony analysis.

Also, the extinction of a species is not the end of the story: insect taxonomy simply transfers to archaeoentomology with attendant information loss.

Systematists need not only to communicate with nature conservationists but also to appraise them. Otherwise, Nero will play at the computer while forests burn, and sentiment rather than science will decide the issue.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

A revived opportunity for fetal research

Diana W. Bianchi, Merton Bernfield and David G. Nathan

One of President Bill Clinton's early decisions was to lift the ban on federally supported research on human fetal tissue. Under what circumstances can research on this material be justified?

DURING the four decades that have followed the US Supreme Court's landmark *Roe vs Wade* decision, the debate over abortion has intensified to the point of ferocity, culminating recently in the murder of an obstetrician. Much of the effort of the anti-abortion movement has been directed against clinicians and against researchers working on fetal tissue. Thirty years ago, a national commission chaired by Kenneth Ryan produced guidelines for research on living human fetuses. The Ryan commission assumed that the tissues of dead fetuses would be treated precisely like those of any dead individual, under the terms of the Anatomical Gift Act.

Indeed, dead fetal tissue did not become a subject of contention until 1988 when the first results of treatment of people with Parkinson's disease using fetal adrenal glands were announced. In the minds of 'right-to-life' adherents, the reports conjured up the image of a massive abortion market fuelled by the need for fetal parts. The Bush administration responded by prohibiting the use of dead fetal tissues for research of any kind in institutions supported by federal funds. Now that the Clinton administration has swiftly and decisively reversed the ban, the time is right to discuss the scientifically and ethically appropriate uses of this material.

We believe that the use of discarded fetal tissue for research and/or therapy should be to increase knowledge of human development and/or improvement of the human condition. Acknowledgement of the unique and non-trivial nature of the material should be mandatory. The review panels now assembling to evaluate research proposals must ensure that human material is essential, and animal or substitute models should be used for preference when possible.

Although pre-implantation conceptuses and zygotes fertilized *ex utero* have contributed to our understanding of phenomena occurring during early human embryogenesis, aborted fetal tissues are more available and so provide new opportunities for clinical research, which if encouraging will stimulate more basic research in human development. What are these opportunities?

Human development

Relatively little is known about the cellular events dictating differentiation in hu-

mans. The use of fetal tissue can provide insights, for example the characterization last year¹ of two distinct subsets of pluripotent stem cells from human fetal bone marrow. Individual fetal cells with specific immunophenotypes were found to differentiate either into haematopoietic precursors or into the stromal cells capable of supporting them. The work, if confirmed, could eventually lead to clinical application in the treatment of aplastic anaemia and malignancy. Other research should help to show why the fetus is particularly susceptible to human teratogens.

Detailed study of placentation, fetal organs and physiology can now be more easily related to human development. Of particular interest is the occurrence of confined chorionic mosaicism, a condition in which the fetal karyotype is discordant with the placenta, which has been described in 1 per cent of cases of chorionic villus sampling performed for prenatal cytogenetic diagnosis². Fetal trisomy with subsequent selective chromosome loss has already been demonstrated to have significant effects on fetal well-being³, and may be the initiating step in the development of disorders associated with uniparental disomy⁴. Material obtained from terminated fetuses will allow cytogenetic and molecular characterization of the relationships between the fetus and its extra-embryonic tissues, contributing to an understanding of events preceding various clinically recognized conditions.

Human therapy

Although the transplantation of fetal neuronal tissue into the basal ganglia of patients suffering from Parkinson's disease has received media attention, the potential for fetal cells to act as a vector for gene-transfer therapy will probably have a more significant long-term impact. Retrovirus-mediated gene transfer into transplantable cells has been proposed for several conditions, but treatment of immunodeficiency caused by adenosine deaminase (ADA) deficiency appears imminent. Transfer of the human ADA gene into haematopoietic stem-cell precursors has been achieved in murine models⁵, and long-term expression of the transplanted human ADA gene in rhesus monkeys has now been demonstrated⁶.

Fetal cells are inherently preferable to

their adult equivalents for transplantation because they are immunologically immature and retain the ability to proliferate. Fetal material is transplantable either as an organ or as a cellular suspension. Cellular transplantation can be performed either pre- or postnatally, the techniques are straightforward, cells can be manipulated in culture, and there is the possibility of extended storage by cryopreservation⁷. In France, haematopoietic stem cells derived from human fetal livers (7–12 weeks' gestation) have been transplanted into four unrelated second- and third-trimester fetuses with inherited immunodeficiencies or haemoglobinopathies. In three of the four cases, engraftment was successful, resulting in amelioration of symptoms⁸.

Human fetal tissue has already been used to treat disorders ranging from inborn errors of metabolism to neurodegenerative diseases for all age groups. In France, fetal liver transplantation has been performed for the past 16 years, with encouraging, if preliminary, results⁹. There are improvements in patients with advanced Parkinson's disease who received human fetal ventral mesencephalic implants^{10–12}. Experiments can now be designed systematically to study factors related to the clinical success of these transplants. The results are likely to have application in a range of degenerative disorders. □

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The dark side of molecular biology

The general enthusiasm for the fortieth anniversary of the structure of DNA should not blind us to a few blemishes on the otherwise fair face of molecular biology.

THE season of celebration of the fortieth anniversary of Watson and Crick's account of the structure of DNA, now past its peak, has been conducted with great restraint. Nostalgia by the great, good and elderly has been delicately muted and mostly amusing. Equally, triumphalism of the dotty kind has not been in evidence at all; nobody, for example, has let slip the belief that the present knowledge of genetics is so certain and detailed that the future of the human race can now be predicted. Nor has anybody suggested that its survival can be assured by pumping into interstellar space countless replicas of the human genome under the slogan "Peace through panspermia" or something like it. All that is eminently creditable.

Indeed, the research community's self-restraint may have been carried too far. It has been mostly left to outsiders to remark that knowledge of the structure of DNA has made possible and has enforced a change of thinking about the nature of living things. The process has been demystified and brought within the gambit of the familiar laboratory discourse. Perhaps the most telling lesson of the past forty years has been the recognition that very different forms of life are built around essentially similar molecular mechanisms. All species are discovered to have more in common with each other than their differences would suggest. Humble *Drosophila* is a better genetic model of human beings than people have ever had before.

The potential benefits are also huge. A proper understanding of the history of the surface of the Earth is not the least of them. But it is also probably the case that, in molecular biology, the gap between basic research and its application is smaller than in any other field of technology, and may even have shrunk to zero. That is why the imaginative imitation of life processes will bring great benefits to our successor generations, who will no doubt give 'biotechnology' a name that will reflect more accurately the grandeur of its promise.

Two other features of this anniversary have not been sufficiently remarked upon. One is the speed with which the revolution in biology has been effected. The past forty years have been technically foreshortened by a succession of powerful techniques for unravelling the behaviour of seemingly complicated macromolecules. You seek a way of making DNA that is complementary to a molecule of messenger RNA? Then find the natural enzyme that allows some cells to do just that. (The name "reverse transcriptase"

then coins itself.) In retrospect, the techniques that have given molecular biology its pace have hung on the principle that life, which has evolved the manipulative techniques by which it is itself sustained, must also embody the techniques for which laboratories cry out.

It has also been remarkable, in the past few weeks, that people who had not been born in 1953 have been conspicuous at the anniversary celebrations. That is not so much a sign that molecular biology is a young person's game, but rather a proof of how great a magnet for young people's enthusiasm the structure of DNA has proved to be. On this occasion, at least, the schools and even the general newspapers have not failed the research enterprise.

Even so, it is a pleasing feature of the recent celebrations that the young and mostly confident practitioners of molecular biology have been able to rub shoulders with its inventors — not just with Watson and Crick, but with their mentors, people like Perutz, for example. All too often, the antecedents of revolution are separated by more than a human lifespan from their fruition. Younger people have a natural tendency to believe that the science they practise has been extant for the whole of time. It is both salutary and stimulating to show that it has roots in people's minds.

So what can be amiss with a torrent of intellectual change as imaginative and potentially as beneficial as that represented by the present condition of molecular biology? On the face of things, nothing. Are not the journals full of new discoveries, some of them important? Is it not now plain that molecular biology stands in relation to all the life sciences as atomic theory does to the physical sciences? (The old argument, fashionable in the 1960s, whether molecular biology is a separate discipline or, instead, an ingredient of every other has long since been settled in favour of pervasiveness.) Yet there are blemishes, some of them important, that deserve wider attention.

First, there is a somewhat technical point. For all its success, molecular biology is still preoccupied with enumeration — the enumeration of the molecular components of cells and of the distinctive organisms to which they belong. The practitioners are still largely in the state of new recruits to any government's army, who will be required at an early stage to learn by rote the names of the standard machine-gun issue.

This is the spirit in which people are now collecting the details of new genes, and of

new nucleotide sequences to go with them; new proteins and their amino-acid sequences; and novel membrane protein molecules, channels or receptors as they may be. None of this implies that molecular biology is simply a higher form of botanizing as practised a century ago; on the contrary, people's descriptions of their experimental methods are replete with numerical information about such things as the concentrations of reagents. With some exceptions (the assembly of tubulin into microtubules is one), molecular biology does not collect quantitative information about the rates of processes happening in cells that physical chemists would consider essential to understanding. Nor is it much concerned with exceptions to the rule that, to every effect, there is one principal cause.

That, given that only forty years have passed, may be forgivable. It is less easily defensible that the practitioners appear to think less deeply about the meaning of the present abundance of data than is the case in many other fields of science. To be sure, coffee-breaks in molecular biology laboratories are as marked by speculation as in any other field, but the published literature gives the impression that its authors are more concerned with the correctness of their observations than with their significance. Especially because of all the evidence that different species, and different processes within a single organism, use variants of similar molecular mechanisms to carry out specified tasks, those with the good fortune to have the time to think about the data accumulating in the literature would probably reap a rich harvest of understanding.

The explanation of the unreflective state of molecular biology is easily accounted for by its third contemporary characteristic: competitiveness. There can never have been a field of research in which the likelihood that people would make similar discoveries almost simultaneously has been as great. The anxiety to publish quickly, if unreflectively, is reinforced by the reward system in research, which links the award of research grants and promotion to people's publications records. This is an old diagnosis of other ills, of course, but it adds both tension and anxiety to a field of science that would be different, and perhaps even more fun, otherwise. But of course, if Watson's *The Double Helix* is to be taken seriously, competitiveness has been the norm since 1953.

John Maddox

Collapse and growth

Robert F. Curl

THE hardest part of the buckminsterfullerene story to accept has always been the formation of this highly symmetric molecule in condensing carbon vapour. If extremely directed efforts by conventional organic chemistry cannot yet make this molecule, its spontaneous formation out of chaos stretches credulity. von Helden, Gotts and Bowers, on page 60 of this issue¹, and Hunter *et al.*, in the *Journal of Physical Chemistry*², report the first experiments which demonstrate that more open and disorganized carbon clusters collapse into a fullerene. Bowers's team at the University of California, Santa Barbara, proposes such collapse as the means by which buckminsterfullerene is synthesized.

The problem is not just that buckminsterfullerene forms, but that it does so with such efficiency. A carbon plasma is created in an electric arc struck between two graphitic electrodes; up to a fifth of the carbon vaporized ends up as buckminsterfullerene³. Scientists have responded to the challenge of this result: the mechanism considered by von Helden, Gotts and Bowers is the fifth to be put forward. The first was proposed by Smalley⁴, who believes that the spherical molecule is produced by the growth of open sheets of pentagons and hexagons. The minimum energy path for such growth incorporates at each step the maximum number of pentagons consistent with a rule that each pair of pentagons should be separated by a pair of hexagons. This prescription he calls the "pentagon road"; it leads inevitably to buckminsterfullerene.

Alternative

Heath⁵ has proposed an alternative "fullerene-road" scheme in which fullerenes are formed in the size range of 30–40 carbon atoms and grow by the addition of small carbon radicals. The growing fullerenes eventually become buckminsterfullerene which, being the most stable structure in its size region, should be relatively inert to further radical attack.

In the other two mechanisms, buckminsterfullerene is formed by a combination of specific precursor carbon clusters. Goeres and Sedlmayr⁶ propose the six-fold combination of naphthalenic C₁₀ units; Wakabayashi and Achiba⁷ propose a mechanism that starts with the combination of a naphthalenic C₁₀ unit with a ring C₁₈.

The trouble with these latter two mechanisms is that naphthalenic C₁₀, comprising a pair of benzenic rings sharing a common edge, is much higher in

energy than monocyclic C₁₀ and should be present only in extremely low concentration. Generally, any mechanism that relies on reactions between specific pairs of medium-sized clusters will be flawed, because their specific presence in the plasma will be too small to account for the high yield of the experiments. The observed carbon-cluster distributions exhibit no extremely special medium-sized clusters.

The fullerene-road mechanism seems to have a similar failing; there must still be sufficient small radicals around when the smaller fullerenes have developed for growth to continue. As the formation of these smaller fullerenes seems to require extensive annealing of more disorganized clusters, they may form at a relatively late stage of clustering. One may doubt whether the small carbon radicals are still present. High buckminsterfullerene yields have been obtained in a heated pulsed laser vaporization⁸ where there is no continuous source of new small radicals as there might be from the arc.

Moreover, buckminsterfullerene can be made with metal ions incorporated in its interior; the internal cavities of small fullerenes are too small to hold metal ions. In the fullerene-road mechanism, the metal ion would have to be taken up on an open cluster early on during growth, and the fullerene shell would then have to grow around the metal.

The pentagon-road scheme does not have this problem. The highly reactive pentagon-road fragments, if they form at all, would form at an early stage of clustering when many small radical species remain. The reliance on highly reactive and as yet unobserved species in the pentagon road is certainly not a fatal objection: the bulk chemistry of combustion is carried by highly reactive free-radical species never present in high concentration. More of a problem is the fact that the mechanism does not spell out a route to producing C₇₀ fullerene. But under the appropriate conditions, C₇₀ can be produced in high yield.

There is no evidence ruling out either the pentagon-road or fullerene-road schemes, but the field is at a state where new suggestions for the fullerene formation mechanism are welcome. The Californian team submits in this issue that high yields of C₆₀ and C₇₀ can be explained in terms of the collapse of larger clusters of carbon atoms with various structures. Such a collapse had been hinted at in molecular dynamics computer simulations⁹. The new experimental results in this issue and by Hunter *et al.*

now confirm that this amazing thing does actually happen, at least for cluster ions near C₄₀⁺.

Bowers's group has previously shown that distinct isomers — chains, single rings, double rings, shells and so on — of carbon cluster ions can be separated in a variant of gas-phase chromatography. That group and Hunter *et al.* now adapt this method to observe the fate of mass-selected carbon clusters accelerated into a drift tube filled with inert gas. They find that the less stable isomers of the larger clusters, for example C₄₀⁺, heated by collisions with the inert gas, collapse and rearrange themselves into more stable fullerene shells.

Annealing

Bowers's team imagine that near a carbon arc, the less stable isomers grow to become larger than C₆₀ or C₇₀ and then collapse to the stable structures as heat from the arc anneals them. Not only have similar events now been seen with smaller isomers, but the mechanism relies on species known to be abundant in the carbon plasma. Also, it is well established⁸ that efficient synthesis of buckminsterfullerene requires high temperatures, which can be understood in terms of the annealing mechanism.

For the scheme to work, it is necessary that the trigger point or detonation temperature for collapse of the non-fullerene cluster depends rather sensitively upon cluster size. Obviously, if collapse to a fullerene occurs before the cluster has accumulated 60 atoms, buckminsterfullerene cannot be produced. If the clusters grow too big before they collapse, they will be unable to shake off enough smaller carbon fragments to get back down to 60 atoms. One can speak loosely of a "non-fullerene cluster", but there is likely to be a variety of such clusters and the collapse size must depend on the chemical bonding in the cluster. A corollary of the mechanism is that either not too many kinds of clusters must be involved or the collapse size has to be fairly independent of the cluster structure.

The viability of the new mechanism can be examined in several ways. The dependence of the collapse of ions of

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various structures into fullerenes upon size and ion energy can be investigated. From such studies, the detonation temperature as a function of cluster size might be estimated. Laser vaporization in a heated tube⁸ provides a means for synthesizing fullerenes by carbon condensation under controlled conditions. The composition of the material produced under a variety of conditions may plausibly be compared with simulations based on the data obtained from the

drift-tube collapse. As a simple example of a prediction that can be tested using the laser vaporization tube set-up, the collapsing model predicts (as does the pentagon road) that the ratio of the C₇₀ yield to the C₆₀ yield will be high when the tube temperature is low, and *vice versa*. Time and further work will tell. □

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activation initiates a cascade of diverging biochemical pathways that conspire to produce an effect (cell division, in this case); and it seems to require that multiple kinases phosphorylate a small number of substrates, which, in turn, feed signals into a smaller number of Ras activators. Accordingly, each step in the signalling funnel would have to be relatively permissive in signal reception yet specific in transmission.

Two events helped lead to resolution of this puzzle. First, there was the identification of the 'Src homology' domains⁹ SH2 and SH3 and the recognition (see refs 10 and 11 for reviews) that these regions are binding sites for specific tyrosine phosphoproteins (for SH2 regions) and specific proline-rich motifs (SH3 regions). SH2 and SH3 regions have been identified in a large number of proteins involved in signal transduction. Their profound significance lies in their restricted permissivity, meaning that an SH domain within a single enzyme can be 'plugged in' to a number of different upstream activators.

The second event was the identification of 'adaptor proteins', of which Grb2 is now the best known (others include Shc, Crk and Nck). These adaptors were proposed to connect other signalling proteins together through SH2 and SH3. Given the semi-permissive nature of interactions between SH2 and tyrosine phosphoproteins, and (possibly) between SH3 and its targets, this presented a daunting number of possible protein-protein combinations.

The new papers cut through these complexities and provide a remarkably clear and simple picture of SH2- and SH3-mediated interactions (see figure). In this scheme receptor activation results in autophosphorylation, creating a binding site for Grb2, as described previously¹². The new insight¹⁻⁸ is that Grb2 associates with Sos1, and thus recruits it to the activated receptor in the plasma membrane where Ras activation is presumed to take place.

Grb2 binding of Sos1 *in vitro* facilitated the rapid mapping of the sites of interaction: the SH3 domains were clear candidates for interaction on the Grb2 protein,

because mutations in these domains had been detected in genetic screens in worms, and indeed both SH3 domains of Grb2 are required for high-affinity binding¹. Attention was drawn to the proline-rich regions of Sos1 as potential SH3 binding sites by the mapping of other SH3 binding sites to proline-rich

SIGNAL TRANSDUCTION

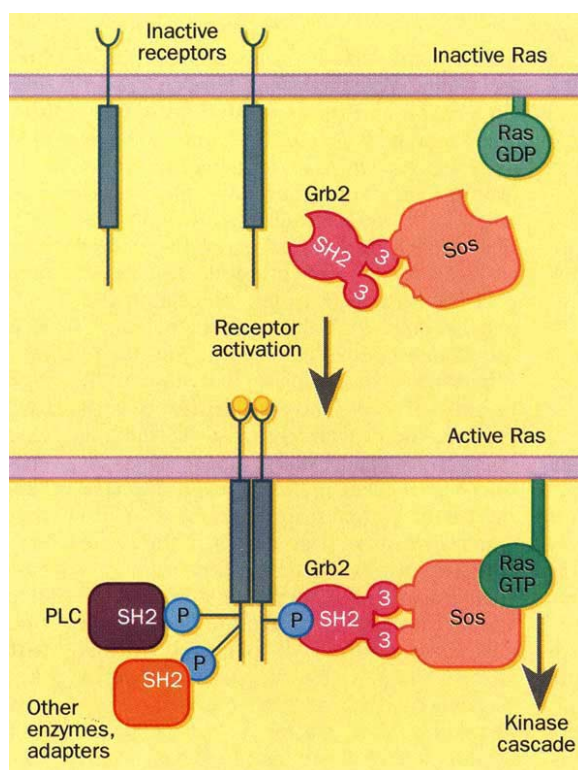
How receptors turn Ras on

Frank McCormick

THROUGH a remarkable convergence of biological and technical insights, a collection of papers (four in this issue of *Nature*¹⁻⁴, three in *Cell*⁵⁻⁷, one in *Science*⁸) describe events by which signals are transmitted from a receptor tyrosine kinase to Ras through specific protein-protein interactions. All eight papers describe the role of Grb2 (Sem-5 in *Caenorhabditis elegans*, drk in *Drosophila*) in recruiting a Ras activator to the receptor to form a stable complex. The Ras activator is the mammalian homologue of *Drosophila* Son of sevenless, referred to as mSos1, which functions as a guanine-nucleotide-releasing protein in converting inactive RasGDP to the active, GTP-bound form by nucleotide exchange^{1,7,8}. Recruitment of Sos1 facilitates Ras activation^{1,3,6-8} and subsequent signal transmission down the Ras-dependent kinase cascade (see figure). Also, the sites of interaction between Grb2 and Sos1 have been mapped^{1,2,4}, and the biological consequences of Ras activation by Sos and Grb2 documented^{1,3}. These papers, the fruits of extensive collaboration among most of the groups involved, provide a comprehensive description of the first steps of this critical pathway along which messages pass from the cell surface to the nucleus.

The flow of information from tyrosine kinases to Ras proteins had been anticipated by analysis of signalling in mammalian cells and genetic analysis of signalling pathways in worms and flies. These studies defined a pathway that is at the heart of growth control in higher eukaryotic organisms, one which is so highly conserved that its components are functionally interchangeable between mammals, flies and

worms. Within any one cell of an organism, it is likely that components of the pathway are used to detect multiple signals, Ras acting as a kind of turnstile through which the signals must pass.



From cell-surface receptor to Ras and beyond. Grb2 binds to activated receptors by the SH2 domain, and to Sos by the SH3 domain. Receptor-associated Sos provokes GDP-GTP exchange on Ras, triggering a cascade of serine-threonine kinases that send trophic signals to the nucleus. PLC, phospholipase C.

One puzzling aspect of this notion was that a variety of tyrosine kinases (receptors and non-receptors) and possibly other activators, such as certain G-protein-coupled receptors, could all funnel signals into the same, highly conserved recipient, Ras. This would seem to run counter to the idea that receptor

domains¹³. These regions are indeed the targets of Grb2-SH3 interactions, although exactly which sequences in Sos1 bind to which SH3 domain is not yet completely clear.

These data raise a number of specific questions, and some more general issues. Specifically, how is Ras activated by Grb2-Sos binding? This interaction is not dependent on receptor activation; it clearly occurs *in vitro* using recombinant proteins, and Grb2-Sos complexes can be detected in non-activated cells. Binding of Grb2-Sos to activated receptors seems to increase the affinity of Grb2-Sos interaction, but guanine nucleotide exchange of Sos1 itself looks to be independent to the binding state of its chaperone adaptor protein⁷.

This leaves open the possibility that the act of recruiting Sos to the plasma membrane triggers Ras activation by generating an increased local Sos concentration. In support of this, overexpression of *Drosophila* Sos1 in mammalian cells causes transformation¹. So it seems that Ras can be activated by merely increasing the total cellular concentration of activator, without requiring receptor-dependent modification. The general idea that receptor activation recruits enzymes to their membrane substrates (Sos1 to Ras in this case) is not a new one: the appearance of lipid-metabolizing enzymes (PI3' kinase and phospholipase C for example) in receptor complexes adjacent to their substrates may be a related phenomenon.

More generally, it is likely that Grb2-Sos can be 'plugged in' to many other receptor systems, as already discussed. Indeed, Egan *et al.*¹ go beyond the association between the epidermal growth factor (EGF) receptor, Grb2 and Sos, and show that, in Src-transformed cells, the Shc adaptor protein replaces the EGF receptor as the phosphotyrosine target and presumably achieves the same end point (activation of Ras). Identification of the precise mechanism by which Shc connects to Src, and its generalization to other non-receptor tyrosine kinases, cannot be far away.

At first sight, a number of proteins

(PI3' kinase, phospholipase C, p120-GAP, for example) implicated in transducing signals from receptors to Ras are left out of the picture. But this does not mean they are not involved. It is conceivable, for example, that the accumulation of RasGTP following Grb2-mediated activation may be compromised by high levels of GAP activity, so that effective signal transmission occurs only when GAP is inhibited. It is also possible that Grb2 or Sos proteins are modified by other signals emitted from activated receptors to compromise or increase their ability to couple. We can now test these possibilities.

Another protein that appears to be left out of this picture is RasGRF, a mammalian Ras activator with a more restricted tissue distribution than the Sos

proteins¹⁴. This protein does not contain the proline-rich sequences that couple Sos to Grb2 and hence to receptor tyrosine kinases. Presumably, RasGRF is recruited to the membrane by a different mechanism in response to incoming signals, the nature of which has yet to be determined. A corollary is the prediction that cells expressing only the Sos variety of Ras activator may be able to respond only to signals transmitted through tyrosine kinases. One thing is certain. If research continues at this pace, stoked as it is by a network of collaborations, these issues should be resolved in months rather than years. □

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COSMOLOGY

Dark matter strikes again

Bernard Carr

DARK matter has been invoked in many astronomical contexts. There is good evidence that it is associated with individual galaxies as these seem to have dark haloes which extend well beyond the visible stars. There are also good reasons for associating it with rich clusters (groups of several hundred galaxies) as these seem to have a collective halo, possibly formed from the material in the original haloes of the constituent galaxies. If one believes the prediction of the inflationary cosmology, that the total density of the Universe must be very close to the critical value above which it recollapses, then there must also be unclustered dark matter as even the dark matter in galaxies and clusters has too small a density to account for this. The evidence for unclustered dark matter is less equivocal but there are some indications from recent studies of large-scale cosmic structure. On page 51 of this issue¹, Ponman and Bertram report a discovery which suggests that the presence of dark matter in yet another context — small groups of galaxies — so that the dark-matter problem rears its head again.

Ponman and Bertram's work concerns the compact group of four galaxies called HCG62. The first step in the argument is their finding that HCG62 is enveloped in a large cloud of hot gas which extends well beyond the galaxies themselves. The evidence for the gas comes from observations by the Rosat satellite, which is able to detect its X-ray emission. The evidence for the dark matter comes from the temperature of the gas, which is found to be about 10⁷ K. This is because the temperature is determined by the

strength of the gravitational field, and the visible material alone (the combination of the gas and the galaxies) does not provide enough gravity to explain such a high value. It has been known for many years that rich clusters contain a lot of gas and dark matter, but Ponman and Bertram's result shows that these features apply more generally.

There is a precedent for this discovery in a similar result reported in February by Mulchaey *et al.*² for the compact group HCG92. These authors also found evidence for hot gas and dark matter extending beyond the visible galaxies, and their work was itself the subject of a News and Views article³. However, there are important differences between the two cases. The first concerns the metallicity of the gas, the abundance of elements heavier than lithium. In both cases the metallicity is low compared with the solar value; this indicates that the gas is mainly primordial, with a small admixture of material processed by stars and then ejected from the galaxies. However, whereas it is only 6 per cent of the solar value for HCG92 (the smallest value ever found for the intragalactic medium in a group or cluster), it is 15 per cent for HCG62.

The second difference concerns the ratio of the baryon mass (that is, the mass in the form of stars and hot gas) to the dark mass (which may be in the form of exotic particles like neutrinos or axions). This is about 4 per cent for HCG92, which is comparable with the ratio of the average baryonic density, derived through models of cosmological nucleosynthesis, to the critical density. If one believes that the total density of

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the Universe must be very close to the critical value, this implies that the baryon fraction in compact groups is representative of the mean cosmological value. However, the baryon fraction is at least 13 per cent for HCG62, which is significantly larger than the global value, so the situation is not as simple as it first appeared. Indeed, Ponman and Bertram question Mulchaey *et al.*'s result on the grounds that the baryon fraction in HG92 may be bigger if one considers a larger region around the group.

There are therefore two problems with HCG62: there are not enough baryons to explain all the dark matter but there are too many to be consistent with the simplest picture of cosmological nucleosynthesis. In fact, the second problem already applies for rich clusters⁴, because these tend to have baryon fractions in the range 20–30 per cent, which is even larger. This puzzle was highlighted by Frenk at the UK National Astronomy Meeting, in Leicester last month, in a paper⁵ discussing what he termed the “baryon catastrophe” for the Coma cluster. This is one of the closest rich clusters and the first one for which a dark-matter problem was identified (see figure). In this case, Rosat observations suggest that the ratio of baryon mass to total mass within 3 megaparsecs is about 25 per cent, which is five times as large as the cosmological ratio in the standard model.

There are various ways out of this dilemma. Perhaps the cosmological density is well below the critical value (despite the anticipation of theorists) or perhaps models of cosmological nucleosynthesis must be revised (as some people have already asserted). More palatable to cosmologists would be the possibility that the gas in groups and clusters has become more concentrated than the dark matter. The difficulty with this is that it is not obvious how the extra baryon concentration would come about — cooling would be unimportant in the outer parts of clusters, so the gas would not naturally sink inwards as in a so-called cooling flow. Indeed many astrophysical processes, such as the spreading out of gas by galactic winds and supernovae, would *decrease* the local baryon fraction.

Another possibility is that the gas density may have been overestimated for some reason. This would be reasonable if the gas were highly clumped as the X-ray intensity per unit mass would then increase. In this context, it may be relevant that the degree of baryon catastrophe seems to increase with metallicity (indicating that cooling may be an important factor). However, for Coma at least, one must reconcile this with the fact that the X-ray emission seems to be rather smooth. Recent Rosat observa-

tions do show evidence for substructure on some scales⁶ — and this is important because it indicates that larger-scale cosmological structures built up hierarchically — but not the smaller-scale clumping required to remove the baryon catastrophe.

There are several other interesting aspects of Ponman and Bertram's paper. One important point is that they have

mass, not only in the group as a whole, but also in its individual members. Another interesting consequence of the drag effect is that, within a further billion years, all the galaxies should have merged to form a single central elliptical galaxy surrounded by an extensive gaseous and dark halo. Ponman and Bertram suggest that there might be many such ‘fossil’ groups in the Universe

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Baryon catastrophe? The central region of the Coma cluster of galaxies, which is 170 megaparsecs away and around 3 megaparsecs in radius. Gas between the galaxies contains too few baryons to account for all the apparent mass but too many to be consistent with the simplest Big Bang models. (Distances assume Hubble's constant $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$.)

been able to determine the temperature profile of the gas and this shows that, within the central 75 kiloparsecs, the temperature decreases as one goes towards the centre of the cluster. This suggests that the gas is undergoing a cooling flow, as is expected anyway as the gas density inferred from the X-ray luminosity requires that the cooling time be less than the age of the Universe within that region. There is good evidence for cooling flows in rich clusters and around individual galaxies⁷ but this is the first example of their occurring in compact groups.

Another interesting point is that measurements of the velocity dispersion of the galaxies in HCG62 suggest that they have drifted inwards by a factor of about three compared with the dark matter. This could be explained as resulting from the dynamical drag of the surrounding gas provided that the galaxies start off with individual dark haloes. (The dynamical friction time varies inversely with the galaxy mass and must be less than the cosmological time.) Thus HCG62 provides evidence for dark

and, if so, that these might be detectable by Rosat. Indeed candidates for such sources may already exist among the cooling-flow galaxies found by the Einstein satellite⁸.

So Ponman and Bertram's paper impinges on a number of important issues: the prevalence of the dark matter, the existence of cooling flows, and the evolution of galaxies and clusters. But perhaps its prime message is the crucial role that Rosat is likely to play in our understanding of these issues. □

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Life and times

THE recovery of no fewer than eleven kinds of filamentous microfossil from 3,465-million-year-old rocks in Western Australia (J. W. Schopf *Science* **260**, 640–646; 1993), shows that life was not only in existence a few hundred million years after the planet formed, but was thriving: rarely does one find single fossils of this antiquity, let alone entire communities. The fossils represent bacteria and presumably photosynthetic (and therefore oxygen-producing) cyanobacteria little different from those a billion years younger. But, as Schopf asks, if such diverse communities were present at this early date, why did their members remain essentially unchanged for so long?

Waste disposal

THE growing end of a neuron receives the necessary materials by axonal transport from the cell body. The metabolic products of the activity at the growth cone come back down the axon by retrograde transport and home in on lysosomes in the cell body, or in some cases are processed by proteases in the cytosol. P. J. Hollenbeck now illuminates the mechanisms by which the unwanted proteins are despatched from the growth cone (*J. Cell Biol.* **121**, 305–315; 1993). The vehicles that carry them back, he finds, are membrane-encased vacuoles, which take up fluorescent dextran, introduced into the cell, and convey it to the lysosomes in the cell body. If the neuron is allowed to take up similarly labelled materials from the outside world by endocytosis at the growth cone, all the fluorescence again appears in the same kind of vacuole, travelling down the axon. This is a finely tuned mechanism for responding to the imperatives of the law of conservation of mass.

Slit decision

THE number of games that can be played to illustrate wave–particle duality is proliferating. Besides diffracting light through material slits (Young's slits), one can diffract atoms through gratings composed of immaterial light waves, atoms through slits composed of other atoms and, now, light off a pair of atoms suspended in a vacuum in a magnetic field (U. Eichmann *et al. Phys. Rev. Lett.* **70**, 2359–2362; 1993). The alert might recall that various groups have made unusual, tenuous 'crystals' out of small numbers of ions suspended in a magnetic field; the pair of mercury ions in the new experiment constitutes the simplest such crystal. So the diffraction of laser photons becomes a novel form of Bragg reflection, which effect showed originally that X-rays are electromagnetic waves and that atoms in solids line up in ordered arrays. Such is progress.

Planet X: a myth exposed

Gerald D. Quinlan

THERE is no reason for the planetary system to end at Pluto. Astronomers have long debated the possibility of a more-distant tenth planet, usually referred to as Planet X. But the scant dynamical evidence requiring the existence of this planet is largely refuted in a new analysis by Myles Standish of the Jet Propulsion Laboratory¹.

The history of the hypothetical tenth planet begins with the discovery of Neptune². By the late 1830s the existence of this unseen planet had become undeniable because of discrepancies approaching one arcminute between the observed and computed orbits of Uranus, attributable to gravitational perturbations. The discovery of Neptune in 1846 within one degree of its predicted position was without question the greatest triumph of celestial mechanics at the time. The discovery reduced the discrepancies in Uranus's orbit to the level of arcseconds, comparable with the noise in the observations, but that did not stop some astronomers from predicting a planet beyond Neptune, of a few Earth masses. A long series of searches beginning with Lowell in 1905 culminated with Tombaugh's discovery of Pluto in 1930.

Pluto appeared tiny right from the start; it is now known to have a mass only 0.2 per cent of that of the Earth, far too small to have caused the irregularities in the orbits of Uranus and Neptune attributed to Planet X. A dedicated group of astronomers have maintained that a tenth planet is required. Some have proposed other reasons for Planet X, blaming it for causing periodic comet showers and mass extinctions on Earth, or for stealing Pluto from Neptune and reversing the motion of Neptune's satellite Triton. Observational surveys by Tombaugh, Kowal and the Infrared Astronomical Satellite (IRAS) would almost certainly have revealed another Pluto or larger planet, but their failure to do so has not stopped many predictions from being made^{2,3}, including two more in just the past year^{4,5}.

Standish shows in his new work¹ that the dynamical evidence for Planet X is readily explained by uncertainties in planetary ephemerides. The construction of an ephemeris involves processing and evaluating the wide variety of data on planetary positions, choosing a dynamical model for the Solar System, and varying the adjustable parameters to give computed orbits that best fit the observations. Planet X's supporters point to three ephemeris difficulties suggesting a tenth planet: the Uranus

observations cannot all be made to fit, and the residuals show systematic trends; a few known sightings of Neptune before its identity was established are difficult or impossible to fit; and predictions of Neptune's orbit have tended to be inaccurate. Standish examines the first two difficulties; the third is not surprising given Neptune's long orbital period, 165 years (so that not even one orbit has been completed since it was discovered).

The Uranus difficulties result mainly from the use of an incorrect mass for Neptune. The masses of the jovian planets are now known accurately from the Voyager missions, but in the past they were not, and much of the speculation on Planet X has been based on residuals from the Jet Propulsion Laboratory's ephemeris DE200 and its predecessors, which used a Neptune mass in error by 0.5 per cent. Standish has constructed a new ephemeris with the correct mass and finds that this reduces the root-mean-square Uranus residuals by about 20 per cent and, more importantly, removes the systematic trends. There remains a strong positive bias in the right-ascension residuals from 1895–1905, but Standish has traced the majority of these to a single catalogue of the US Naval Office 9-inch transit telescope (data from two other sources do not show this bias); an observational error such as an incorrect equinox correction is a plausible explanation.

Regarding the predisccovery sightings of Neptune, Lalande's in 1795 is said to differ from ephemeris predictions by seven or more arcseconds, but Standish shows by extrapolating two ephemerides backwards that the predictions are uncertain by at least several arcseconds; combined with the scatter in Lalande's observations this makes the discrepancy rather dubious. The discrepancy for Galileo's observation in 1613 is larger if true, but Galileo's notes are ambiguous, and, moreover, Standish and Nobili have found another Neptune observation by Galileo, this one agreeing with ephemeris predictions (personal communication).

Accurate planetary observations being collected today will in time lead to more stringent tests for a tenth planet. These observations are valuable irrespective of Planet X, because planetary ephemerides are needed for other purposes such as spacecraft navigation and pulsar data reduction, and are instrumental in precise tests of theories of gravitation. But for now the search for Planet X is futile.

The search for smaller objects in the

outer Solar System has only just started, however. Dynamical limits⁶ allow a few tenths of an Earth mass in a belt of planetesimals beyond Neptune. This belt, named after Kuiper who suggested it would be left over from the formation of the planets, has been much-discussed as a possible source of short-period comets⁷. In 1987 David Jewitt instituted a systematic search of the outer Solar System for faint, slow-moving objects. The effort was rewarded with the discovery⁸ last August of the faint smudge (7,000 times fainter than Pluto) which he and Jane Luu tagged 1992QB₁ (they later named it Smiley, after le Carré's spy). Four months' observations of QB₁ reveal an orbit that would be expected for a Kuiper-belt object, although there is still considerable uncertainty in the eccentricity. QB₁'s estimated diameter of 200 km puts it at one tenth the size of Pluto: much larger, and the whole distinction between planets

and minor planets would be thrown into doubt. There is now news^{8,9} of a second possible Kuiper-belt object, 1993FW, also found by Jewitt and Luu. The discovery of even a few of these objects could tell us much about the early history of the Solar System, perhaps more than we would learn from the elusive (or illusory) Planet X. □

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SCHISTOSOMIASIS

Proselytizing with immunity

Edward J. Pearce

THE reality of schistosomiasis is that at least as many people are infected today as ten years ago — this despite the availability of the wonder drug praziquantel. It was against this gloomy backdrop, and in the knowledge that disease resistance, disease severity and drug efficacy are contingent upon the immune response to the parasites concerned, that immunologists got together earlier this year* to assess the state of play in understanding and tackling the disease.

The outlook, however, is not altogether depressing. The meeting provided a sparkling overview of the very real advances in getting to grips with the immunoregulatory and immunopathological mechanisms involved, and in vaccine development. Even the sceptics were left with no doubt that the production of an effective vaccine against schistosomiasis is not only necessary, but possible.

There is, it is clear, more than one way to make a vaccine against schistosomiasis. Recombinant schistosome glutathione-S-transferase, which confers only a partial resistance to infection, induces a potent anti-fecundity immune response that dramatically reduces the number and viability of eggs produced by female parasites, and so reduces the risk of transmission and the pathology associated with infection¹ (A. Capron, Pasteur-Lille). The anti-pathology

effects of this vaccine are most striking in calves and patas monkeys, both of which were essentially protected from disease when unvaccinated controls developed severe lesions. There was, too, an intriguing report of unprecedented levels of immunity induced in rodents, and partial protection in baboons, by a recombinant surface-antigen (rIrV5), which shares homology with myosin II (M. Strand, Johns Hopkins). The plan is to take these vaccines into phase I human trials in the near future.

There is also progress to report in identifying immune effector mechanisms, an area in which research on schistosomiasis has been central. Amongst those working with human populations in endemic areas there is accord that the intensity of reinfection following treatment, as assessed by faecal egg counts, is negatively correlated with levels of immunoglobulin E (A. Dessein, INSERM; D. Dunne, University of Cambridge), lending support to the view that IgE protects against schistosomes. (Incidentally, the time-honoured use of faecal/urine egg counts to assess infection intensities may be on the way out; many groups are now planning to use circulating adult antigen to provide specific information on worm burdens.) On the subject of responses mediated by the Th2 subset of CD4⁺ cells, the story of the eosinophil grows more intriguing with news that these cells are a major source of interleukin-5 and possess IgA receptors (M. Capron, Pasteur-Lille).

Another approach to vaccine development is the elicitation of Th1-like responses, as illustrated by the report that immunity in mice immunized with attenuated parasites is entirely dependent on the Th1 cytokine, interferon- γ (ref. 2; A. Wilson, University of York). The demonstration that endothelial cells can be activated by IFN- γ to kill schistosomes (S. James, NIAID) raises the prospect that these cells, which line the pulmonary capillaries (a dangerous bottleneck in schistosome migration), have a part in the IFN- γ -mediated effect. The take-home message here is that both Th1- and Th2-stimulating vaccines have the potential to be protective.

During the egg-laying stage of infection, Th responses in mice are highly Th2-like, suggesting that pathology, which is Th dependent, is mediated by Th2 cytokines. This concept was discussed in light of observations that IL-2 (D. Boros, Wayne State; A. Sher, NIAID), the α form of tumour necrosis factor (ref. 3; Boros; J. McKerrow, UCSF) and IL-4 (Boros; Sher) are all involved in granuloma formation; further, in mice, high TNF- α levels seem to be predictive of severe disease (D. Colley, Centers for Disease Control). Studies of the antigens involved in anti-egg Th responses show egg proteases to be particularly immunogenic (B. Doughty, Texas A&M).

The involvement of IL-10 in the inhibition of macrophage anti-schistosome effector functions (James), and the Th1 down-regulation which accompanies egg-associated Th2 responses, came in for some debate. This is a critical area — experimentally, the suppression of Th1 and CD8⁺ cell activity makes mice with schistosomiasis more susceptible to experimental vaccinia infection⁴ (in these mice, virus persists specifically in granuloma fibroblasts; Sher). These data bear on the correlation between hepatitis and schistosomiasis, and (possibly) on how the human immunodeficiency virus is dealt with by helminth-infected individuals. The extent to which Th1 responses are down-regulated in infected patients, who clearly are mounting anti-parasite Th2-like responses, remains unclear, although some studies have indicated less of a suppression than that seen in infected mice (Dunne; C. King, Case Western Reserve).

The belief that pathology in schistosomiasis is wholly egg-centred requires reassessment in light of the report of 'capillarization' of hepatic sinusoids in infected mice before the onset of egg production (J.-A. Grimaud, Pasteur-Lyon). Other news on pathogenesis is that fibroblast stimulating factor 1, a cytokine first described in schistosomiasis, stimulates deposition of extracellular matrix (D. Wyler, Tufts). The relationship between a failure to down-

* *Fundamental Immunology and its Application to the Study of Schistosomiasis*, Sharm El Sheikh, Sinai, Egypt, 31 January–11 February 1993.

modulate anti-schistosome immune responses during infection, and the likelihood of developing severe life-threatening disease, was the subject of much comment, as was the importance of exposure *in utero* to regulatory idiotypes and/or antigen to prevent severe disease following infection (Colley).

Schistosomes use host molecules for their own devices. In this context, the discovery that *Schistosoma mansoni* uses TNF- α as a stimulus for egg production³ was given added significance by the observation that the site where these parasites achieve sexual maturity, the portal venous system, is particularly rich in this cytokine (McKerrow). On this subject of direct parasite-host interactions, egg- and larval-stage surface glycoproteins have been shown to carry lacto-*N*-fucopentaose III; this contains the Lewis-X trisaccharide, a ligand for P-selectins (D. Harn, Harvard), raising the possibility of direct communication between schistosomes and host cells through adhesion molecule interactions. An exciting implication, by analogy with certain tumour cells that express Lewis-X and metastasize to the lungs and liver (the two major systemic organs through which schistosomes migrate), is that this sugar provides a means for the parasite to find its way around the host. Lacto-*N*-fucopentaose also activates B cells from schistosome-infected mice (in an antigen receptor-independent manner), to make IL-10, a cytokine implicated in the development of the Th2-like response evident in infected animals (Harn).

So, experimentally, progress is tangible. But if there was a sense of excitement at the meeting about these new results, there was also a sense of urgency. In some countries things are getting worse rather than better — for instance, the current epidemic of schistosomiasis in Senegal is characterized by the emergence of unexpectedly severe disease and the alarming failure of praziquantel⁵. The hope is that unusually fruitful conferences such as this one, in which a course in the basics of immunology preceded seminars on the latest developments, and in which the participants in most part came from areas badly affected by the disease, will show us the way forward. □

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Max in a complex affair

Paul Freemont

ON page 38 of this issue¹, Max meets DNA courtesy of Stephen Burley and colleagues. Max is a transcription factor, a protein that binds to specific DNA target sites and controls the polymerization of RNA from adjacent DNA templates. There are surprises to be found in the striking images of the complex between Max and its DNA target sequence, and in those surprises are hints about the interactions of Max with several other proteins, which are directly involved in the control of cell growth.

In all living cells, proteins have to recognize and bind to specific DNA sequences, recognition generally resulting from the interactions of particular amino acid side-chains with the nucleotide bases of specific DNA sequences^{2,3}. Quite a number of protein–DNA complexes have now been determined to atomic resolution by X-ray crystallography, which has led to the charac-

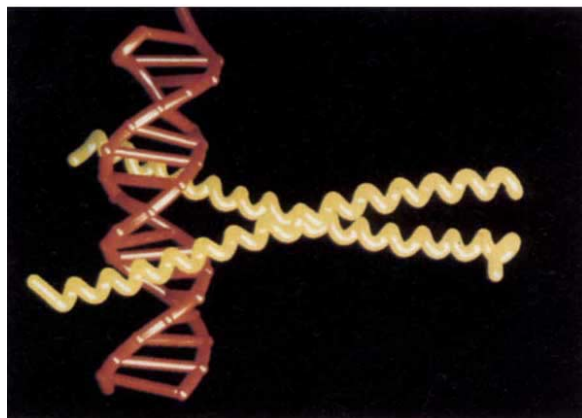
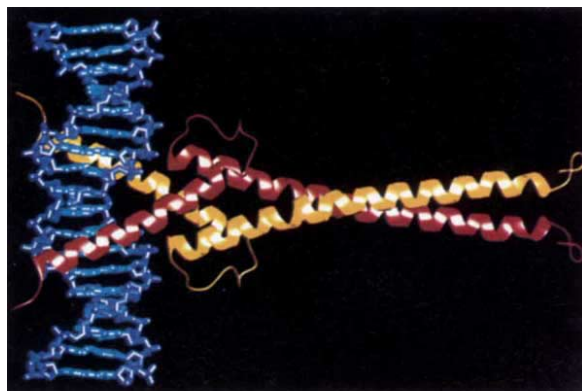
terization of a variety of discrete protein modules which mediate these interactions³. What Burley's group has now done is to add a new protein structural motif to this list by solving the cocrystal structure of the basic/helix-loop-helix/leucine zipper (b/HLH/Z) domain of Max bound to DNA at 2.9 Å resolution.

The helix-loop-helix motif (HLH) is found in a variety of dimeric eukaryotic transcription factors and, as the name indicates, it was first characterized on the basis of two amphipathic α -helices connected by a loop⁴. Amino-terminal to the HLH region, a further helical sequence of basic residues is found and, in some members, a well-conserved leucine zipper is present after the HLH motif. Each of these regions has been the subject of extensive mutagenesis and biochemical experiments, from which it seems that the basic regions mediate

DNA binding, and the HLH and zipper region promote dimerization (see refs 4 and 5).

The structure of the Max homodimer–DNA complex¹ shows that the b/HLH/Z motif is made up of two lengthy α -helices separated by a loop, and not several helices as the motif's name would suggest (see figure). The amino-terminal helix (25 residues) includes residues for the basic region and the first helix of the HLH, whereas the carboxy-terminal helix (43 residues) comprises the second helix of the HLH motif and also the leucine zipper motif. The two helices are in contact with each other near the loop region (10 residues), specific contact residues being conserved throughout the HLH family.

Most unusual, however, is the way in which the Max homodimer (and by inference other b/HLH/Z proteins) recognizes its DNA target sequence. The two basic amino-terminal α -helices sit in the major groove so that they are perpendicular to one another on opposite sides of the DNA duplex; they resemble nothing so much



Representations of the crystal structures of the complexes between DNA and Max (b/HLH/Z motif) and GCN4 (bZIP motif) showing the similarity in DNA interaction for both types of domain. Top, the Max–DNA structure of Burley and colleagues¹. The α -helices are drawn as ribbons (red and yellow) and the DNA duplex is shown in blue. Below, the GCN4 bZIP–DNA structure⁶. The α -helices (yellow) are drawn as smooth continuous curves with the DNA coloured red.

as a pair of short chopsticks. These helices extend from a novel four-helix bundle structure which is stabilized by conserved hydrophobic residues that form a hydrophobic core. Four specific DNA base contacts are made by residues from the basic helix, as are a large number of phosphate contacts spanning the entire recognition sequence. These interactions make sense of the high degree of sequence conservation observed within the basic helix for different b/HLH/Z proteins, as all the conserved residues make either base or phosphate contacts.

Most intriguing, though perhaps not surprising, is the similarity between the Max-DNA structure and that of the yeast transcriptional activator GCN4 bZIP-DNA complex (ref. 6, see figure). The DNA-binding domain of GCN4 also has a basic helical region and a leucine zipper (bZIP) but without the helix-loop-helix that characterizes the HLH family. Like Max, GCN4 also binds to its DNA target sequence as a dimer, with the two basic helices acting like a pair of chopsticks gripping opposite sides of the major groove of the DNA duplex. Curiously, the leucine zipper structures for both proteins are almost identical, and one could view Max as a bZIP protein but with an extra pair of 'prongs'.

However, the Max basic helices are shorter than those for GCN4 by some two turns of an α -helix, and the angle between the two chopsticks interacting with DNA within each complex is slightly different (about 14° down the DNA helix axis in Max and 18° in GCN4). The different interhelical angles observed between GCN4 and Max might reflect the need for both proteins to recognize different DNA target sequences. But they probably result from the nature of the dimerization interfaces, which are very different for each protein. When compared to the bZIP family, the HLH family has evolved a much more substantial interface for dimerization, as illustrated in the Max structure, where dimer contacts are made between four α -helices as opposed to two for bZIP proteins. This distinction suggests that dimer stability (and to some extent inflexibility) are key features of the b/HLH/Z family, which is consistent with the fact that *in vivo* they can form reasonably stable homo- and heterodimers not bound to DNA⁵. In contrast, the bZIP domain seems to be very flexible; the basic helix is unfolded in the absence of DNA, resulting in a potentially more versatile DNA-recognition element which could adopt different conformations on different DNA sequences⁷.

There's more, however, to the new Max cocrystal structure than the revelation of a novel structural domain for specific DNA interaction, for it also

provides insights into the mechanisms of differentiation and malignant transformation. *In vivo*, Max is the specific partner for the c-Myc oncoprotein (see ref. 8). Furthermore, heterodimerization of Max with c-Myc mediates DNA binding by c-Myc, which is essential for both the normal and the oncogenic activity of c-Myc⁵. Max has been found tightly associated with a new partner Mad/Mx1^{9,10}, which points to Max being at the centre of a network of factors whose relative concentrations dictate preferential heterodimerization and thereby control transcriptional activity. So the Max homodimer-DNA structure provides a first glimpse of the potential molecular interactions between Max, c-Myc and Mad/Mx1, and as such provides pointers as to the combined role of these proteins in controlling cellular growth.

What next? Before too long we will probably be presented with the structures of other HLH-DNA complexes including the upstream regulatory factor,

a Myc-Max heterodimer and perhaps other Max heterodimer complexes. Armed with these and other detailed structural pictures, we may at last be able to get a handle on some of the many and complex eukaryotic transcriptional control mechanisms at the molecular level. That is a prospect to savour. $\square$

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ASTRONOMY

Shocking behaviour of young stars in Orion

John Dyson

STAR-FORMING regions in dense molecular clouds offer rich hunting grounds for astronomical observers and theorists interested in exotic dynamical phenomena. Generally, such phenomena are interpreted in terms of the interaction between steady winds from young stars with material in the surrounding cloud. Now, on page 54 of this issue¹, Allen and Burton present spectacular infrared photographs of active star-forming regions in the Orion molecular cloud which reveal the cloud's deep interior. The intriguing structures they find may be evidence that sudden explosive events play the dominant role in at least some dynamical phenomena, and that molecular clouds therefore have a rightful place in the current perception of the interstellar medium as one subjected to assaults of extreme violence.

Discovered some 40 years ago, Herbig-Haro (H-H) objects are deceptively nondescript patches of emission seen in projection against dark cloud backgrounds. Their spectra show that the radiating gas is excited by hydrodynamical shock fronts and that shock velocities of up to a few hundred kilometres a second are involved. Corroborative evidence of this comes sometimes from the spectral widths of the lines, and sometimes from observation of movement of the patches, revealed in sequences of photographs. The objects come

in various shapes and usually, though not always, seem to be associated with a nearby star. As diverse evidence also indicates that the stars found in star-forming regions lose mass either from their surfaces or from surrounding accretion disks, a causal connection between stellar mass loss and the H-H objects has become a widespread article of faith.

In principle, the generation of any supersonic disturbance of a high enough Mach number (velocity divided by the local speed of sound) in a molecular cloud will produce H-H-like spectra, provided that the shocked gas can radiate sufficiently well. It is therefore not surprising that many variants of the interaction between stellar winds and molecular clouds have been explored to account for the differences from one H-H object to another.

It was realized very early on that interactions involving isotropic stellar winds had serious inherent problems involving energy. Were the kinetic energy of the wind to be converted by the shock into radiant energy, the localized H-H objects could expect to take up only a small fraction of the wind's energy, in which case the radiated power far exceeds that available. Ingenious mechanisms were invoked to collimate the winds and so focus their energy. A few years ago, it was found² that nature had done this already: young stellar objects have

jets associated with them. Jet-cloud interactions can generate localized H-H objects in a variety of ways, ranging from the production of internal shocks in the jet to radiation behind the shock-wave systems at the front of the jet as it bores its way through the surrounding cloud. Considerable attention is currently being given to the production of H-H objects by jets that precess or in which the jet velocity varies. Curiously, how stars produce jets is an open question.

One of the earliest attempts to explain H-H objects was in terms of the motion of fast-moving 'bullets' of material through the molecular cloud³. The radiating gas — the H-H object — originates in shocked cloud gas behind the bow shock on the leading face of this cosmic missile. This model fell out of favour for numerous reasons. For example, the origin of the missile was far from clear; and as detailed structures of many H-H objects were elucidated, the jet interactions described above seemed a much better proposition. Allen and Burton now argue¹ that, at least as far as their observational data are concerned, the projectile model best fits the facts.

They note that the molecular hydrogen emission reveals long tail-like structures which project back to a more or less common origin in the vicinity of well-known stellar residents of the Orion molecular cloud (see cover photo). The ends of the tails furthest away from the 'origin' are not visible in molecular hydrogen but are bright in the light of forbidden transitions from singly ionized iron. This latter emission is a well-known indicator of shock activity for shock velocities of up to several hundred kilometres a second. Allen and Burton also point out that there are families of H-H objects in the Orion cloud that are bright in the forbidden lines of singly ionized oxygen^{4,5} and that all such knots have corresponding iron emission features. The oxygen lines, studied in detail, have spectral widths up to 400 km s⁻¹, which must indicate actual gas velocities of this magnitude⁵.

Allen and Burton propose the following model. A few thousand years ago, some violent explosive phenomenon occurred which hurled numerous bullets ('shrapnel') into the molecular cloud with velocities probably in excess of 400 km s⁻¹. The bullets swept up cloud material through the bow shocks on their leading faces, producing the iron and oxygen emission behind the shocks. The velocity widths of the lines will range roughly up to the bullets' speed. Molecular hydrogen is rather fragile (in interstellar terms), and is destroyed by shock waves with velocities greater than about 50 km s⁻¹ and cannot exist behind the leading shock. However, bow shocks become wrapped around blunt obstacles

and become much weaker the further away they are from the obstacle head. Molecular hydrogen could survive and be excited by passage through these extremely oblique shocks.

The origin of the bullets is obscure and a real understanding of their energetics is needed to pin it down. A rough estimate by the authors suggests that some scaled-up version of so-called FU Orionis events might be plausible. These are quite well-known occurrences in which stars of a certain class liberate a considerable amount of energy through an astrophysical trauma involving instabilities of the accretion disk. Alternatively, Allen and Burton note that a supernova would have enough kinetic energy to power the bullets, although there is certainly no other direct evidence for one having occurred in this region. On the positive side for such a picture, observations of (astrophysically) recent supernovae such as Cassiopeia A or even the Crab show that supernova explosions are extremely fragmented.

These various possibilities are extremely exciting, but a great deal of follow-up observation and modelling needs to be done. It is crucial to develop realistic shock models to see whether plausible parameters can be found which give the correct behaviour of the observed emission. It may perhaps be premature to jettison continuous wind interactions completely. Although Allen and Burton, probably correctly, discount the possibility of a collection of individual jets each powering a molecular hydrogen 'finger', excitation by a precessing jet may be plausible, though this depends on whether continuous emission can be maintained as the jet precesses. Recent work on sporadic jet outbursts by a precessing jet⁶ shows considerable promise for explaining families of H-H objects. Finally, there are puzzling aspects of the oxygen emission, notably that it is particularly strong at essentially zero velocity⁵. This is very hard to fit into a projectile model.

Whatever the resolution of these problems, Allen and Burton's observations show that many surprises are in store as modern observational techniques enable us to probe into hitherto unexplored regions of interstellar space. □

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Monopoly money

EVEN the most advanced modern economies cycle regularly between boom and slump. This reminds Daedalus of an ecology with relatively few interacting species. The populations of predators and prey both rise and fall violently. By contrast, a complex ecology with many different species shows a deep stability. Individual populations may still fluctuate, but the overall balance of the community remains steady.

What lack of diversity destabilizes an economy? Daedalus reckons it is the single currency. The EC's goal of a single currency is thus exactly wrong, as you might expect. Instead, it should achieve financial unification by making all its currencies legal tender in every member state.

The experiment would be highly instructive. The present national currencies are mutually well insulated. Their rates of exchange are quite artificial, being set by government interest-rate policies, and are easily exploited by parasitic speculators. But once pounds and deutschmarks and francs and lira were all competing against each other in every high street, their exchange rates would always reflect what each currency could actually buy. Even the biggest speculators could not distort them, or make much profit by doing so.

Ecological theory holds that no two species can occupy exactly the same niche. So Daedalus expects that the various currencies would diversify into different 'economic niches'. In doing so, they would highlight the various roles that money plays in society: a medium of exchange or of speculation, a store of value, and so on. One likely niche corresponds to the scavenger species in an ecosystem. This currency would dominate declining industries and regions (the British pound seems a natural candidate). By depreciating against the other currencies as needed, the scavenger currency would insulate them from this local depression. A society with n independent currencies should be $\sqrt{n}$ times as stable as one with a single currency.

Even better, governments could no longer cheat their citizens by inflation. At present, any government can simply print currency and spend it, enriching itself while impoverishing its citizens. But in a multicurrency society, the citizens could hedge away from such trickery. A currency that started inflating strongly would rapidly be abandoned for all purposes, except that of paying tax to the government printing it.

David Jones

Amorphous polymer granules

SIR — Poly-*R*-3-hydroxybutyrate (PHB), a hydrophobic storage polymer found in bacteria, is used commercially as a biodegradable thermoplastic¹. It is stored *in vivo* in granules whose structure has long been debated. Isolated PHB is highly crystalline, and it was originally believed that the native granules were also crystalline. It is now clear, however, that the polymer is stored in cells as a mobile elastomer². Many

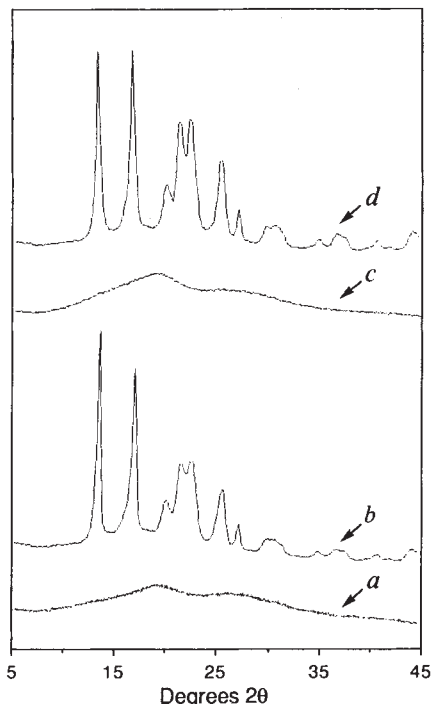


FIG. 1 Wide-angle X-ray diffraction patterns for a, whole *A. eutrophus* cells; b, crystalline PHB powder; c, artificial amorphous PHB granules; d, artificial granules dried overnight at 125 °C.

hypotheses have been advanced to explain the metastable amorphous state of the polymer *in vivo*³, most recently that it is simply the result of the slow nucleation kinetics that are operative for small particles^{4,5}. We now provide experimental support for that explanation by preparing artificial granules which effectively contain only PHB and are virtually indistinguishable from native storage granules.

The essence of the *in vivo* kinetic model is that polymer that is newly biosynthesized in an amorphous mobile form within the granule will only crystallize as a result of homogeneous (spontaneous) nucleation. In the absence of heterogeneous nucleation or granule coalescence, an ensemble of 0.25-μm granules should have a crystallization half-life of more than 1,000 yr at 30 °C, whereas bulk samples of the polymer

quenched to 30 °C from the melt typically crystallize within a few minutes. It should therefore be possible to reconstitute stable amorphous PHB granules *in vitro*^{6,7}.

Accordingly, a solution of PHB (99.9% pure) in CHCl₃ (5% wt/v) was sonicated at 20 kHz with 20 vol of an aqueous solution of cetyl trimethylammonium bromide (CTAB, 5 mM). The resulting emulsion was dialysed exhaustively over 48 h against additional 5 mM CTAB to remove the organic solvent. Laser light scattering measurements confirm that the remaining PHB is particulate, with median diameter 0.3–0.4 μm. Nycodenz gradients show that the PHB particles have density 1.18 g cm⁻³, the same as native granules and pure amorphous PHB; crystalline PHB powder has a density of 1.241 g cm⁻³ (ref. 8). The surfactant-coated, amorphous PHB granules were collected by centrifugation for further studies.

The wide-angle X-ray diffraction patterns obtained from pastes of either artificial granules or PHB-rich whole cells of *Alcaligenes eutrophus*⁹ show only an amorphous halo, whereas crystalline PHB powder gives a series of sharp peaks (Fig. 1). In addition, the NMR spectra of the reconstituted granules show the same signals and temperature dependence as the polymer contained in whole cells (Fig. 2)², implying essentially identical mobility properties. No signals are detectable from CTAB associated with the artificial granules. Isolated PHB powder, which is about 70% crystalline, has no ¹³C-NMR spectrum at these temperatures. Artificial amorphous PHB granules prepared as described are stable in aqueous suspension at 30 °C for at least 6 months.

We have thus established that PHB mobility *in vivo* can be explained solely on the basis of the intrinsic material properties of the polymer. Figure 1d demonstrates that by a simple drying treatment the artificial amorphous granules can be caused to coalesce and returned to their original highly crystalline

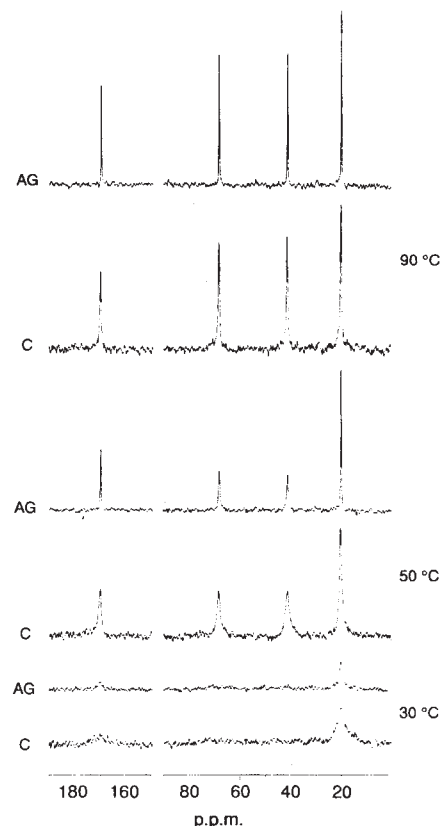


FIG. 2 Natural abundance 100-MHz ¹³C-NMR spectra at three temperatures of artificial PHB granules (AG) and whole cells of *A. eutrophus* (C). Artificial granules were collected by centrifugation (33,000g), resuspended in D₂O and examined in a NMR tube containing a d₆-benzene capillary.

state. Presumably, the removal of surface water destabilizes the surfactant-polymer interactions that keep the granules physically separate. The findings we report here have interesting implications for the preparation of polymer coatings.

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Scientific Correspondence

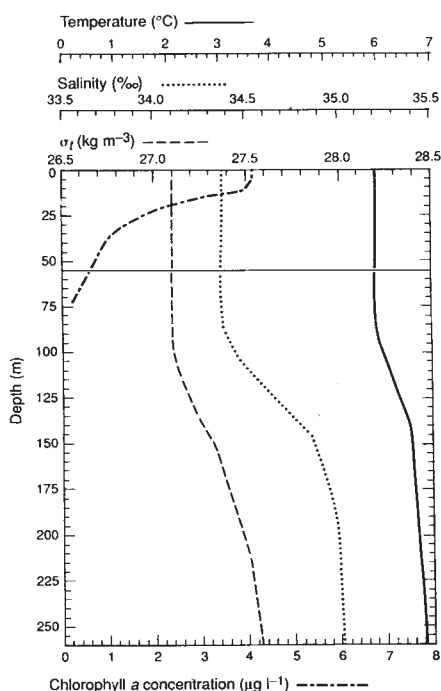
Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

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Spring blooms and stratification

SIR — I would like to comment on the paper by Townsend *et al.*¹, who reported that spring phytoplankton blooms occurred in the absence of vertical stratification of the water masses. Balsfjorden, near Tromsø (69° 20' N), is a 60 × 3-km-wide fjord with a bottom depth of 210 m in which the spring bloom takes place in homogeneous water columns². In these areas, freshwater run-off from melt water starts to increase early in May and peaks in June. Hence no stratification of the water masses resulting from lowered salinities can be observed. The spring bloom starts during mid March and culminates at the end of April, following the yearly spring increase in incident radiation, in the absence of water column stratification throughout.

Similar results have also been ob-



Vertical profiles of temperature (°C), salinity (parts per 10³), water column density σ_t (kg m⁻³) and chlorophyll *a* concentration (µg l⁻¹). The profiles were taken at Malangsdupet (69° 52' N, 16° 46' E) in April 1990 using a Neil Brown Mark III CTD sonde system. Chlorophyll *a* concentration was measured at selected depth intervals, typically every 5 m from the surface (starting at 0 m) after an estimation of micro-scale vertical chlorophyll distribution with an *in vivo* fluorometer. Horizontal line, computed "critical depth". Chlorophyll *a* concentration was measured by the fluorometric method after extraction in methanol. The dominant phytoplankton species were *Phaeocystis pouchetii*, *Chaetoceros socialis* and *Thalassiosira nordenskiöldii*.

tained from Skjomen, a fjord close to the polar circle, and for Ullsfjorden, north of Tromsø^{3,4}. During 1984–90, cruises to the Barents Sea have shown that the spring blooms may progress in unstratified water masses (H. C. E. & A. Evensen, manuscript in preparation). Statistical tests clearly show that the biomass increase was correlated to the yearly spring increase in radiation, and not to physical properties of the water, such as temperature, salinity or density.

The figure shows a typical bloom from open coastal areas approximately 60 km west of Tromsø. The sampling at the station was performed approximately 2 weeks before the culmination of the spring bloom. During model runs simulating the onset and the progress of the spring bloom the "critical depth"⁵ was calculated to be about 55 m. The vertical distribution of phytoplankton biomass decreased steadily down to about 100 m. The water masses were nearly isopycnal from the surface to 90 m. Further down, both temperature and salinity increase, causing a smooth increase in density.

It is widely accepted that for a bloom to develop the critical depth must be shallower than the mixed depth⁵. In other words, the null hypothesis is that phytoplankton cannot stratify in the absence of a density gradient (if waters are not shallow). The large bulk of the data that have led to this conclusion originates from areas south of the polar circle. Looking at the spring bloom maximum at several locations along the coast of Norway and at Spitzbergen, from Korsfjord (outside Bergen) to Kongsfjorden

(Spitzbergen), shows that there is a delay in the onset of the spring bloom north of the polar circle⁶. This lag is due to the time lag in the seasonal increase in total radiation, as also pointed out in reports of spring blooms in areas from Lofoten and southwards⁷. However, north of the polar circle no significant delays are observed. The most surprising observation is that the spring bloom occurred almost at the same time in the Tromsø area and at Spitzbergen, approximately 10° further north.

In my opinion, the explanation for this is that until the vernal equinox (21 March) the length of the day increases from north to south in the Northern Hemisphere. After the equinox, day length increases faster further north, that is, the period between 24-hour darkness and midnight Sun is shorter at high latitudes north of the polar circle. This means that day length increases faster at high latitudes, and that primary production is enhanced compared to areas further south.

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Cerebellar flocculus hypothesis

SIR — Lisberger and Sejnowski¹, and Judge in News and Views², discussed what is known about the cerebellar control of the vestibulo-ocular reflex (VOR). The 'flocculus hypothesis' is that a small area of the cerebellum, the flocculus, adaptively controls VOR by regulating signal flow from labyrinths to the flocculus to the relay cells of VOR, and has been challenged on several grounds. But I believe the hypothesis can still be defended in the light of recent knowledge.

The traditionally defined monkey flocculus includes the genuine flocculus and the ventral paraflocculus³. Earlier data contradicting the flocculus hypothesis⁴ were obtained in the ventral paraflocculus⁵. But, when recorded only from those sites where local electrical stimulation evoked lateral eye movement, suggesting their close connection to the horizontal VOR, the behaviour of Purkinje cells is consistent with the flocculus hypothesis^{6,7}.

The characteristic synaptic plasticity in the cerebellum, long-term depression (LTD), an important basis of the flocculus hypothesis, has now been well documented. One reason why Lisberger and Sejnowski assume another synaptic plasticity in the brain stem pathway is that LTD alone does not explain the two-way adaptation, increase and decrease of the VOR gain. However, this argument overlooks the fact that two subsets of input to flocculus Purkinje cells arise from bilateral labyrinths, which modulate out-of-phase to each other and which undergo LTD separately⁸. Whether VOR gain increases or decreases will depend on which subset of input undergoes LTD.

Brain stem synaptic plasticity has also been suggested based on measurements of eye movements and related neuronal responses during VOR before and after adaptation in monkeys⁹ and goldfish¹⁰. However, the evidence is rather indirect. Further, the fact that lesion of the cer-

ebellum including the flocculus in rabbits¹¹, cats¹² and monkeys¹³, or subdural application in rabbits and monkey of haemoglobin (which blocks LTD)¹⁴, abolishes the entire VOR adaptation, raises the question of why brain stem synaptic plasticity does not show up in the absence of cerebellar plasticity.

Finally, Purkinje cell responses inconsistent with the flocculus hypothesis in monkeys⁴ and goldfish¹⁵ have been observed under visual cancellation of the VOR. Such responses would be free of eye-velocity signals, but at the same time would be contaminated by visual signals or higher command signals acting to cancel the VOR. The signal contents of these responses need careful reconsideration before they can be related to VOR adaptation.

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LISBERGER AND SEJNOWSKI REPLY — We are surprised that Ito is so strongly opposed to a model that accounts for his data and ours, and that allows the cerebellar cortex of the flocculus and/or the ventral paraflocculus to be one of two sites of learning in the VOR. We also find that Ito's response to our Letter¹ contains some omissions and errors that undermine his position.

(1) Ito claims that Watanabe⁶ and Miles *et al.*⁴ recorded from different Purkinje cells in different parts of the cerebellum. He fails to mention that the two studies recorded in common from at least two folia in the ventral paraflocculus and he fails to cite the fact that individual Purkinje cells in these folia show the same effects reported by both groups¹⁶. Therefore neither group's data can be discounted and any model of the VOR must account for both sets of results. Our model does, and the 'flocculus hypothesis' does not.

(2) Ito does not mention evidence that the earliest expression of learning in the VOR evoked by an electrical stimulus in the labyrinth occurs only 5 ms after the stimulus, too soon to allow time for transmission through the cerebellum¹⁷. He does not cite the existence of a group of cells in the brain stem that express learning 12 ms after the onset of head motion, before most Purkinje cells in the cerebellum show any response¹⁶.

(3) Ito incorrectly explains why our model requires sites of learning in the vestibular inputs to both the cerebellum and the brain stem. Our model requires two sites of learning so that constant head velocity can generate constant eye velocity. If learning is localized in just one site in the cerebellar or the brain stem pathway, then a constant head velocity generates an eye velocity that

ramps inexorably toward positive or negative infinity. This behaviour is a consequence of the structure of the model and of the fact that the model processes inputs and generates outputs that vary as a function of time. The need for two sites of learning is unrelated to the exact mechanism of learning and, in fact, the form of long-term depression supported by Ito could be a mechanism of learning in our model.

(4) In defending the 'flocculus hypothesis', Ito fails to cite a study done in rabbits showing that motor learning in the VOR can be acquired without vestibular stimulation¹⁸. This study contradicts the 'flocculus hypothesis', which requires activation of vestibular inputs during motor learning.

Our model, although it requires more than one site of learning, accounts for data from multiple laboratories and reconciles observations that had been thought previously to be contradictory. Even for a relatively simple behaviour like the VOR, a biological system must face many constraints that require a complex neural network with the possibility of multiple sites and mechanisms of learning. The challenge is to elucidate the operation of the whole system, not merely to determine the function of an individual structure or an isolated synapse.

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Palaeoclimate sensitivity

SIR — Those studying global warming generally define climate sensitivity as the response of the globally and annually averaged temperature to a doubling of CO₂. Such a quantity is, in theory, well defined, but to assume that major climate regimes in the past arose from such simple forcing is unjustified. To use past climate to infer sensitivity to a doubling of CO₂, as was done recently by Hoffert and Covey¹, is equally unjustified. In their Fig. 2, both the warmer and colder climates considered may have had tropical temperatures approximately the same as the current climate. But the differences in temperature between the tropics and high latitudes were very different. This, in turn, implies that these past climates were associated with very different north–south dynamic heat fluxes in the atmosphere–ocean system.

Such heat fluxes are important in at least two major respects: first, without them, the annually averaged pole-to-Equator temperature difference for the present climate would be about twice as great as it is². Second, given that the Earth's major greenhouse gas, water vapour, varies greatly with latitude and altitude, it is impossible to calculate the net greenhouse effect without knowing where heat is deposited by the dynamic heat transports³. In addition, the atmospheric contribution to this heat transport is at least as great as that from ocean currents⁴, and is dominated by fluxes in winter⁵.

Dynamic fluxes depend on motions which in turn depend on spatial variations in heating. Thus, orbital variations are believed to have caused the cyclic glaciations of the past 700,000 yr (the Milankovitch hypothesis⁶). These variations involve very small changes in globally and annually averaged insolation, but give rise to major changes in the spatial distribution of heating. Atmospheric fluxes are likely to depend on the intensity of the Hadley circulation in the tropics^{7,8}. This intensity, in turn, depends strongly on the displacement of the zonally averaged summer surface temperature maximum from the Equator⁹, and on the meridional sharpness of the zonally averaged temperature maximum⁸. The former is extremely sensitive to orbital parameters, whereas the latter depends greatly on the nature of monsoons. Monsoons clearly depend on the distribution of land and sea in the tropics; this was certainly very different during the mid-Cretaceous period considered by Hoffert and Covey¹. Incidentally, neither of these two factors depends significantly on gross radiative

parameters like CO₂ concentration or solar constant — at least within the range of variation considered in ref. 1.

Hoffert and Covey's conclusion, that the existence of widely varying climatic states excludes the possibility of low sensitivity to doubling CO₂, is only plausible for a one-dimensional Earth. For the real Earth, it ignores factors crucial to climate not included in the somewhat simplistic climate sensitivity appropriate to CO₂ increases. In particular, if an altered distribution of heating produces a large change in dynamic heat flux, then major changes in global climate may occur, even if the sensitivity to changing CO₂ is extremely small. Indeed, if it should turn out that the tropics are thermostatically stabilized^{10–12}, then changes in forcing which are associated with little change in dynamic heat flux are likely to cause very little global change. The fact that the tropics appear to have remained at approximately the present temperature despite major changes in the Equator-to-pole temperature difference (and the associated heat flux out of the tropics) strongly suggests the presence of some stabilizing mechanism for the tropics.

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HOFFERT AND COVEY REPLY — Lindzen challenges our derivation of global climate sensitivity¹ by suggesting that large climate changes could have resulted from changes in poleward heat flow and/or the seasonal and latitudinal distribution of sunlight, that is, by moving heat from one location to another. Such factors could certainly be the pacemaker of climate change, as in Milankovitch forcing. We believe, however, that significant globally averaged temperature change would ensue only if additional feedbacks from slowly responding elements of the climate system altered the global mean radiative forcing.

Palaeoclimate reconstructions indicate

that the meridional temperature gradients decrease, and poleward heat flow increases, as global mean temperature increases^{2–5}. This does not mean that changes in poleward heat flow cause significant warming, however.

The global energy balance must be satisfied. Near-linearity of longwave flux versus surface temperature⁶ makes it unlikely that mere redistribution of surface temperature can affect global mean temperature much by changing planetary cooling. On the solar absorption side, the tropical cirrus shielding cited⁷ by Lindzen is a negative feedback on temperature, whereas high-latitude ice albedo-temperature feedback is positive⁸. Because an increase in poleward heat flow cools the tropics while heating the poles, such heat flow could by itself lower planetary albedo. But how much would the Earth warm?

Fast albedo feedbacks are too weak to change global temperature significantly without global mean radiative forcing. That is why the 'old' Milankovitch theories fail (see below). Melting all the present-day sea ice can only increase global radiative forcing by $\sim 2 \text{ W m}^{-2}$ (ref. 9). Our measured sensitivity of $\sim 2.3 \text{ K per CO}_2$ doubling (4.4 W m^{-2}) gives the corresponding global warming as $\sim 1 \text{ K}$ — too little to explain the $\sim 9 \text{ K}$ warmer Cretaceous without the greenhouse forcing used in our reconstructions. The much smaller sensitivities favoured by Lindzen could account only for a minuscule fraction of Cretaceous and Jurassic warmth.

Regarding the Hadley cell and monsoon conjectures, current research on Milankovitch forcing indicates a major role of feedbacks on the fast-response atmospheric variables (wind, temperature, moisture, snow and sea ice, clouds) from slowly-responding variables (ice volume, ocean currents, CO₂)¹⁰. Orbital forcing driving an atmosphere model alone cannot simulate the initiation of a glacial cycle¹¹, but can do so if the atmosphere is coupled to slow glacial dynamics (ref. 12 and M. E. Schlesinger, personal communication). Another serious problem with purely atmospheric dynamical models is that the hemispheres were out of phase with respect to the orbital precession cycle believed to have triggered the transition from the Last Glacial Maximum (LGM) deep freeze 11 kyr before present to Holocene warmth¹³. There is evidence that insolation changes somehow trigger changes in the ocean circulation, biological productivity and alkalinity carbon pumps regulating atmosphere/ocean partitioning of CO₂, a greenhouse gas^{14–17}. Coupling with slow ice sheet and ocean feedbacks can account for orbitally-paced planetwide changes of temperature and CO₂ recorded in ice cores in ways that

purely atmospheric models cannot.

We used reconstructions of ice sheets and compositions in ice cores to derive the albedo and greenhouse forcing of the LGM at zero solar mean forcing¹. With forcing and response known, whether CO₂ changes lead or lag climate changes¹⁸ is irrelevant for deriving the climate sensitivity of the LGM.

The most convincing test of any theory is comparison with observations. Our objective is to improve models for the prediction of climate change by tests against palaeoclimate data. We reported early findings that the ratio of global mean temperature change to global mean radiative forcing is very similar for two very different past climates. Our analysis has been expanded to meridional response patterns and additional palaeoclimates. Such validations are necessary regardless of one's disposition towards the current generation of global climate models¹⁹. We hope Lindzen supports his ideas with quantitative models whose assumptions can be compared with observations. At this point we find his conjectures implausible when compared with the view that global mean temperature is determined by global mean radiative forcing, including (but not limited to) greenhouse forcing from CO₂ changes.

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In the name of genetics

Dorothy Nelkin

Exploding the Gene Myth. By Ruth Hubbard and Elijah Wald. *Beacon*: 1993. Pp. 206. \$24.

Biology as Ideology: The Doctrine of DNA. By Richard C. Lewontin. *HarperPerennial*: 1993. Pp. 128. \$10 (pbk).

THE Human Genome Project has encouraged, with research funds, studies of the social implications of a rapidly developing science that promises to reveal the essence of life and to predict our future fate. Much of this work, by sociologists, ethicists and historians, has been critical, focusing on several related themes. Science, and in particular genetics, is shaped by social forces and ideological agendas: the history of eugenics is the usual example. Advances in genetics have troublesome ethical implications, bearing on questions of privacy, justice and equity: the key words are genetic discrimination. And the implications of genetics must be seen in a political context, for the use of genetic information is tied to prevailing political and economic ideologies and the interests of social institutions.

These two books also explore these themes, but two of the authors, Hubbard and Lewontin, are neither philosophers nor social scientists but biologists — critics of their own colleagues. How does this influence their perspective?

Hubbard and Wald's agenda is evident in the subtitle of their book: "How genetic information is produced and manipulated by scientists, physicians, employers, insurance companies, educators, and law enforcers." Writing in accessible terms, they offer a broad overview of the science of genetics and its social implications. Their intention is to convince readers that genetics will have a powerful influence on their lives and that scientists cannot be trusted to protect them: "We cannot just sit by as passive worshipers or victims". They describe their book as "a basic survival handbook".

Their task is to explode "gene myths" — that genes are an all powerful basis of health and disease, that biotechnology is the "wave of the future", that science is immune to political and social pressure, that organisms can be explained in terms of their molecules, that chronic conditions such as cancer can be explained in terms of inherited tendencies. Their point is that these myths developed in the political context of inequality in the distribution of power and resources, in the economic context of capitalism with its market pressures, and, above all, in the social context of a science that is controlled by commercial connections.

Scientists, they argue, have interests in defining normality and deviance, health and disease, in terms of genetics rather than social or environmental conditions.

The authors dwell on the conflicts of interest arising from the commercialization of genetics and biotechnology. Scientists, economically linked to their enterprise and preoccupied with patents, cannot be trusted to protect the public interest even when they seem to try. The authors suspect the programme funded by the National Institutes of Health on the social and ethical implications of genetics of "putting the Human Genome Project in a position to supervise what questions are asked". So they conclude with a social policy agenda. Citizens cannot leave critical decisions in the hands of experts. They must be vigilant, informed and directly involved in decisions about the development and application of science.

Lewontin's book, a collection of his essays on the ideological basis of contemporary biology, is more about political philosophy than social policy. He too explodes the myths encouraged by scientific ways of thinking: that it is possible to understand the world in reductionist terms, that there are clear distinctions between cause and effect, that the world is made up of bits and pieces that can be isolated and studied in isolation. These beliefs, he argues, are just as ideological as the holistic world view of a more mystical, premodern age.

Lewontin is highly critical of "biological ideologues" who perpetuate the belief that science offers simple explanations for social problems. Rather, he says that "things are complicated, uncertain, and messy, that no simple rule or force will explain the past and predict the future of human existence". There is, in fact, only a limited relationship between scientific understanding and the social practices that draw support from science. As history shows, science has ideological and social purposes; it can lend legitimacy to social policies, provide legitimation for social inequities and justify power relationships on the basis of natural categories. Lewontin describes how the genetic world-view — deterministic and reductionist — encourages the view that human life is what it has to be. To suggest that everything we are — our health, our wealth, the very structure of

society — is encoded in our DNA is simply to justify the status quo.

Lewontin counters such thinking with an interactionist perspective. Denying the distinctions between nature and nurture and between biology and ideology, he calls for "an entirely new level of causation" based on the complex interaction between the biology of living organisms and their environment. And science too must be viewed as an interaction between scientists and society. Just as science affects society, so scientific priorities and ideas are shaped by social and economic forces. For scientists are social beings, their ideas moulded partly by social experience.

That Hubbard and Lewontin are biologists by profession shows up in several ways. They are especially skilled at popularizing the technical aspects of genetics. But, beyond general references to market forces and social ideologies, they are not inclined to analyse the structural features of a society that lends credence to gene myths. While proposing solutions through public participation, they feel no need to define appropriate constituencies or organizational strategies. Nor do they deal with the real dilemmas of participation that have been the focus of considerable research. And they can gloss over the social forces that contribute to the appeal of reductionist and deterministic ideas. Indeed, my research on images of the gene in popular culture is revealing trends that make me wonder about the results of public participation. Would it encourage decisions that these authors (and I) would really welcome?

Finally, these books are less constrained and more cynical in their criticism of science than those written by nonscientists. Is it because Hubbard and Lewontin are insiders in the scientific enterprise, protected by their credentials and free to debunk their colleagues? Or are they reflecting growing disaffection among scientists with recent changes in their profession? Science, and especially genetics, has moved from academe to the commercial world, tied to private corporations, wedded to profits. As its image as an independent search for truth has changed, scientists have had mixed reactions. Some respond by trying to capture media attention: thus the gene becomes a "master molecule" and the genome the "Book of Man". Others, disillusioned with their colleagues, react with cynicism and dismay. The perspectives of these two books is clearly influenced by the broader tensions in the scientific community over the changing character of science. □

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Against megabuck science

Arnold Pacey

Invention: The Care and Feeding of Ideas. By Norbert Wiener. MIT Press: 1993. Pp. 159. \$19.95, £17.95.

MANY of us were brought up on stories of great inventors and individualistic engineers, and have since had to re-educate ourselves to understand the development of science and technology as a social process, or even to believe that inventions are 'socially constructed'. Of course, there are social processes that need to be appreciated, but the suggestion that everything should be described in these terms seems extreme. It is refreshing, therefore, to see the publication of this book, written nearly 40 years ago, in which an inventor of real achievement acknowledges "an interplay of economic and social forces" and the importance of the "social environment", but also asserts and describes the role of individual insight.

Norbert Wiener's basic view is that the ideas of individuals can trigger the larger processes that lead to innovation by their

influence on the "intellectual climate" in which science, the arts and technology all develop. Sometimes, new ideas involve such radically novel perspectives that the lack of key individuals "in the chain of thought leading up to them . . . could easily have delayed them for . . . a generation". At other times, though, ideas are less dependent on the right person coming along because they are implied by the current state of knowledge or technique. Such ideas tend to emerge "nearly at the same time in many fields of work, among many individuals, in many countries".

Awareness of the role of key individuals leads Wiener to make a series of points about how research is organized. Most notably, he is critical of "megabuck science", meaning the large-scale project carried out by a big laboratory or industrial corporation. Although he concedes that this kind of research is sometimes necessary, he deplores the tendency to regard it as the norm. Scientists working in a team, each looking at a separate facet of a problem, may well throw light on details, but they are no more likely to make fundamental discoveries than monkeys with typewriters.

Stemming from his view of the role of the individual, Wiener has some telling comments to make about patents and

about the nonsense of leaving scientific research at the mercy of markets and the profit motive. Such policies usually mean that long-term potential will be neglected, and will result "in the inability of the scientist to furnish the long-time and deep-lying developments on which the community as a whole . . . ultimately depends". Where the patent system is concerned, Wiener notices how it is manipulated by industrial corporations to the disadvantage of individual inventors, and one of his main aims in the book is to provoke thought about a fairer approach to patents and about comparable rewards for scientists whose discoveries cannot be patented.

Much of this has a bearing on today's concerns, and one must constantly remind oneself that the book was written in 1954 by an author who is best remembered for his writings on cybernetics. His arguments do not seem dated, although the ideas about scientific revolutions put forward by Thomas Kuhn could have helped him along in one or two places. The book is published now for the first time as a result of scholarly study by Steve Joshua Heims of the cybernetics group of the late 1940s. An introduction by Heims outlines Wiener's own experience as inventor of noise filters, an analogue computer and an electrical network concept. It is interesting to see how this experience led Wiener to ignore the conventional boundaries between 'science' and 'technology', especially when he explains how mathematics is an "organ of invention and discovery". The implications of this perspective are that euclidian geometry, newtonian mechanics and the probabilistic theories of Gibbs and Planck are crucial points in the history of invention because of the way they changed the intellectual climate.

This short book might have been more accessible to the lay reader and perhaps generally clearer had it included some extra detail or more specific examples. But Wiener's comments on the corruption he observed in megabuck science are so scathing that he could have been open to a libel suit had he been too specific. Instead, he quotes Benjamin Thompson, Count Rumford, active around 1800, as "the prototype of the scientific adventurers who have beset the present age of great projects. Where the carcass is, you will find the flies buzzing." Perhaps Wiener left this book unpublished because he felt it would start too many battles. Whatever the reason, its appearance now is both useful and welcome — especially for students of science, technology and society. □

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PRESENCE of the past — this chameleon appears in *Prehistoric Animals in the Modern World* by M. Ferrar and F. Pratesi (Prion, £18.95). With over 120 dramatic colour photographs, the book unearths creatures that survive today largely unchanged from their ancestors of millions of years ago.

Facts and fables

P. M. Rattansi

Isaac Newton: Adventurer in Thought.
By A. Rupert Hall. Blackwell: 1992.
Pp. 468. £19.95, \$29.95.

"FORTUNATE Newton, happy childhood of science . . . Nature to him was an open book whose letters he could read without effort", wrote Einstein in the foreword to a 1931 edition of the *Opticks* (1704). Newton's account of his discovery of the true nature of light and colour has, however, as Professor Hall comments in his new biography, "misled posterity down to recent times". The crucial experiment on which he placed so much weight was "largely fictitious, newly invented for its present purpose". Hall guides us through the far more tortuous path that led Newton to his discoveries and explains why not all his many critics can be dismissed simply as benighted traditionalists.

The story of the falling apple, which Newton himself loved to tell in his old age, would place his discovery of the law of universal gravitation in the *anni mirabili* of the plague years of 1665–66 "when I was in the prime of my age for invention". That, too, is unlikely. Newton seems at that time to have accepted a variant of the ethereal vortices that Descartes had set rotating around the Sun to carry the Earth and the planets in orbital motion. Only after he had set to work, at Halley's urging, on the tract that eventually became the first book of the *Principia* did Newton abandon vortices and envisage attractive — and repulsive — forces in central bodies. It was Newton himself, again, who, during his priority dispute with Leibniz, encouraged the notion that the conclusions presented in a classical geometrical garb in the *Principia* were originally derived by him through his fluxional calculus — one more 'fable'. It is now clear that "the published state of the *Principia*. . . is exactly that in which it was written."

These radical revisions of the conventional account by no means diminish our awe at what Newton achieved in mechanics, mathematics and optics. Hall skillfully weaves the historical research of the 'Newton industry' over the past three decades into the biography. Only occasionally does the account seem like a patchwork of notes left over from his editorship of the last three volumes of Newton's *Correspondence*. Sir David Brewster's Victorian biography, first published in 1855, remained the standard account for a long time. It was not wholly superseded until the publication of Richard Westfall's *Never at Rest* in 1980 (a condensed, updated version of

this biography is soon to be published by Cambridge University Press as *The Life of Isaac Newton*). Hall's substantial biography is about half the length of Westfall's and takes account of much of the more recent historical research.

Brewster was provoked by French suggestions that Newton's surprisingly voluminous theological studies indicated the extent to which his nervous breakdown of 1692 had left him mentally crippled. Brewster demonstrated that Newton had been a "searcher in the scriptures" throughout his life. He rejoiced in this

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Roubilliac's bust of Newton. (Courtesy of Trinity College, Cambridge.)

confirmation of the essential harmony of science and religion. Brewster was troubled, nevertheless, by another side of Newton: an alchemical passion that had left behind a hoard of manuscripts, consisting largely of transcripts from alchemical works. The Sotheby sale in 1936 made the contents of Newton's Portsmouth papers much better known and gave rise to Lord Keynes's famous description of Newton as the "last of the magicians". Those papers, too, have been extensively quarried by a number of historians in recent years. What significance does Hall attach to them?

While conceding that "thirty years of study and the writing of one million words must have left their mark on Newton's mind", Hall is inclined, on the whole, to reaffirm his verdict, published in a joint paper in 1958, that Newton attempted to retrieve useful chemical facts from the enigmatic and mysterious terms employed by alchemists to conceal them. It would be quite rash, therefore, to conclude that Newton in any way accepted the truth or validity of the larger claims advanced by the

alchemists. Hall criticizes the flimsy evidence on which, for example, Newton has been depicted as a member of an active alchemical coterie at Cambridge, supposedly including Barrow, the Lucasian professor whom he was to succeed, and Henry More, the Platonist philosopher. With scrupulous fairness Hall also reproduces in an appendix a report by Karin Figala of Munich, who provided him with a summary of her own research on Newton's alchemical studies.

But Figala's conclusions have devastating implications for any portrait of Newton which, like that provided by Hall, is still largely cast in the conventional mould, even if at great pains to depict Newton 'warts and all'. Figala asserts that Newton was no exception to the seventeenth-century tendency to "search for a combination of the exact sciences with magical thought". She believes that in Newton's alchemy we can catch a glimpse of "at least a small part of his attempt to reconcile magic and science". Acceptance of such a reading of Newton's work would move the alchemical (and prophetic) studies of Newton from the periphery towards the centre of his lifelong endeavours. Many of the documents whose recovery we owe to Hall's industry (notably the 'Chemical' notebook and the tract known as *De gravitatione*), and much other evidence that finds a place in his own narrative (the Cambridge Platonist attitude to the mechanical philosophy, for example), would then form part of a different story. Westfall's biography attempted to integrate the theology and the alchemy with what we now see as the genuinely 'scientific' activities of Newton. Here they still appear as regrettable or puzzling distractions.

With its many virtues, this biography is of a piece with Hall's two other recent books — the revised and enlarged edition of *The Scientific Revolution* (Longman, 1983) and the biography *Henry More* (Blackwell, 1990). It is tempting to see these works as belonging to a formidable and sustained effort to return the history of science to where it was before it was invaded by what Hall may see as sociologizing and 'hermeticizing' interlopers. All these books have strengths that justify their place on the library shelf and on every reading list on the history of seventeenth-century science. But whether it is worthwhile — or even possible — to return to the older project, to which Hall himself made so many pioneering contributions, is a different matter. □

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Born free

Roger F. Searle

The Immunology of Human Pregnancy.
By Henry N. Claman. *Humana*: 1993.
Pp. 232. \$59.50.

THE birth of the topic of reproductive immunology is widely acknowledged to stem from a seminal paper by the late Sir Peter Medawar in 1953, in which he recognized and addressed the immunological problems presented by the evolution of viviparity in vertebrates. Important advances in cellular immunology and molecular biology, especially in the past 10 years, have largely clarified the immunological paradox of pregnancy, but not yet definitively explained how the 'foreign' fetus survives to term without provoking maternal immune rejection. The intellectual challenges posed by the maternal-fetal immunological relationship are now particularly relevant: issues such as HIV infection in pregnancy, and the development of immune approaches to birth control and fertility regulation, attract much attention. Recently, this rapidly moving and multifaceted subject has been the focus of several comprehensive state-of-the-art texts such as *Immunology of Pregnancy* edited by G. Chaouat (CRC, 1993) and *The Immune System in Disease* edited by G. M. Stirrat and J. R. Scott (Baillière Tindall, 1992). It is against this background that Henry Claman's review has to be judged.

Claman sets out to explore in depth fundamental questions, but readers expecting such will be left unfulfilled and let down. The book has been written very much with the busy clinician in mind and conveys only the most superficial impression, an approach that is less than satisfactory for the serious researcher or specialist. It is therefore curious that although the author's original impetus for writing was his involvement in the controversial treatment of

recurrent spontaneous miscarriage by immunotherapy, he has largely ignored the topical clinical arena. This is a considerable shortcoming. Disappointingly for a book published in 1993, there are few references more recent than 1991 (and very few 1991 references at that).

On the credit side, the text is highly readable and will easily guide the non-specialist clinician through the complexities of fundamental immunology and provide a useful introduction to the immunology of human pregnancy. The format of the book follows predictable well-trying themes, covering immunological aspects of the placenta, maternal immunocompetence during pregnancy and maternal responses to fetal antigens. The style is relaxed and informative.

Topics of current intense interest such as the expression and regulation of HLA-G class I molecules on trophoblast cell populations at the maternal-fetal interface are mentioned, but inevitably provide a picture from around early 1991. The implications of HLA-G ex-

pression for the outcome of pregnancy are given scant attention and "remain to be explored", which today is simply not true. Similarly, statements such as "the study of cytokines in the placenta is just starting" are nonsensical in light of the rapid advances highlighting the crucial regulatory role that growth factors play in reproductive processes. The chapters on immune disorders of pregnancy and recurrent pregnancy loss, however, are especially informative and Claman is here to be congratulated for summarizing succinctly a large body of data in a balanced and objective way.

The attraction of the book is that it gives clinicians an easily readable albeit superficial overview that will serve as a useful introduction: and even specialists in the field are likely to find useful information in one or more of the chapters. □

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Molecular receptors in the round

Leonard F. Lindoy

Macrocyclic Chemistry: Aspects of Organic and Inorganic Supramolecular Chemistry. By B. Dietrich, P. Viout and J.-M. Lehn. VCH: 1993. Pp. 384. DM148, £61, \$99.

MACROCYCLIC ligands — cyclic organic molecules with a central cavity — are used by nature to bind metal ions in such important molecules as haem, chlorophyll and vitamin B₁₂. Several natural antibiotics are also macrocyclic and work by metal binding. There are good reasons for nature to choose macrocyclic species to bind metal ions — foremost among these is that the binding is usually considerably stronger than for comparable noncyclic ligands.

In addition to natural rings, a lot of synthetic macrocyclic molecules have been investigated over the past 30 years. Many can act as receptors for small molecules as well as different ion types (including anions). Ionic and molecular recognition is a frequent characteristic of such host-guest interactions, aided by a cavity in the host with fixed or semi-fixed binding sites. Host-guest assemblies of this type fall in the realm of supramolecular chemistry — the chemistry of synthetic receptors and large molecular assemblies that often mimic nature's chemistry. Supramolecular chemistry is now a large and expanding field that is serving to underwrite an emerging new age in chemistry: the age of molecular devices.

In 1987, the Nobel prize for chemistry was awarded to Charles Pedersen, Donald Cram and Jean-Marie Lehn for their pivotal contributions to macrocyclic and supramolecular chemistry. This book is based on a series of lectures on macrocyclic chemistry delivered by Professor Lehn at the Collège de France some years ago. It is divided into two sections: "Macrocyclic synthesis" and "Macrocyclic Complexes — Cryptates".

The first section contains a valuable discussion of synthetic approaches and general strategies for obtaining macrocyclic ligands. In keeping with the French group's formidable record in synthetic macrocyclic chemistry, the content of this section is wide ranging, covering both practical and theoretical aspects. The remaining section focuses on the complexes of both natural and synthetic macrocyclic molecules and presents a panoramic view. Included is an extended discussion of the chemistry of macrobicyclic ligands (the cryptands) and their complexes (the cryptates).

Overall, the book provides an effective way for researchers and graduate students to gain a foundation in the fascinating concepts of the field. It supplies the background for appreciating the many exciting advances currently unfolding. □

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New Journals Issue

This year, *Nature's* annual new journals review supplement will appear in the issue of 7 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared during or after June 1991 and issued at least four separate numbers by the end of April 1993 will be considered.
 - Frequency of publication must be at least three times a year. The main language used must be English.
 - Deadline for submission is the end of May.
- When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title.

For further information telephone Peter Tallack on 071-836-6633 (011-44-71-836-6633 from the US).

Strange kinetics

Michael F. Shlesinger, George M. Zaslavsky & Joseph Klafter

The once abstract notions of fractal space and time now appear naturally and inevitably in chaotic dynamical systems and lead to 'strange kinetics' and anomalous transport properties. An understanding of this kind of dynamical behaviour should provide insights into, for example, turbulent fluid dynamics and particle random-walk processes.

It is now widely appreciated that complex, seemingly random behaviour can be governed by deterministic nonlinear equations. Such complex deterministic behaviour has been termed chaotic. But rather than being a harbinger of the demise of random processes, nonlinear dynamics has opened up a host of new challenges for statistical physics. These challenges focus on characterizing, predicting and controlling the evolution of complex nonlinear processes in space and time. The statistical nature of the problem stems from the possibly intricate, hierarchical and fractal nature of trajectories in nonlinear dynamical systems, and the concomitant extreme sensitivity of the solutions to initial conditions, variation of parameters and system energy.

Here we will mainly investigate hamiltonian (energy-conserving) dynamical systems that exhibit such rich and varied behaviour, particularly looking at problems which were previously overshadowed or unknown. Although it is difficult to predict what will be the future central problem in chaotic dynamics, it is safe to select particle and field kinetics as providing a foundation for problems of importance. An ultimate goal is a kinetic description of chaotic dynamics which will generate insights into the nature of associated random processes (especially random walks), and their link to the theory of dynamical systems. Some aspects of kinetics in hamiltonian systems with chaos will be discussed below in detail. As we will show, simple nonlinearities in the hamiltonian can induce fractal motions with nonstandard statistical properties. We term such behaviour 'strange kinetics'.

Real orbits in dynamical systems are always theoretically predictable because they represent solutions of simple system of equations (for example Newton's equations). Under conditions for dynamical chaos, however, these orbits are highly unstable. Generally for chaotic motion the distance between two initially close orbits grows exponentially with time as

$$d(t) = d(0) \exp(\sigma t) \quad (1)$$

where the rate σ is called a Lyapunov exponent. This dependence holds for sufficiently long times. Local instabilities, described by equation (1), lead to a rapid mixing of orbits within the time interval $\tau_\sigma = 1/\sigma$. Nevertheless, some properties of the system remain fairly stable and their evolution occurs at a significantly longer timescale $\tau_D \gg \tau_\sigma$ as a result of averaging (possibly only partially) over the fast process of mixing, caused by the instability in equation (1). Kinetic equations arise as a consequence of such averaging. The well-known gaussian and poissonian statistics (for diffusion in space and temporal measures, respectively) can under certain conditions give a valid, albeit approximate, description of the apparent randomness of chaotic orbits. It has been realized, however, that behaviours much more complex than standard diffusion can occur in dynamical hamiltonian chaos. For hamiltonian chaos we will emphasize parameter regimes where Lévy statistics (describing fractal processes) apply and strange kinetics rules. Lévy statistics can appear both in space and time, and the fractal processes they describe lie at the heart of complex processes such as turbulent diffusion, chaotic phase diffusion in Josephson junctions, and slow relaxation in glassy materials.

Lévy statistics can be generated by random processes that are scale-invariant. This means that a trajectory will possess many scales, but no one scale will be characteristic and dominate the process. Geometrically, this implies the fractal property that a trajectory, viewed at different resolutions, will look self-similar. One example of a scale-invariant random process is a random walk with infinite mean-squared jump distances $\langle R^2 \rangle$ (the second moment of the jump distance probability, $p(R)$). A finite second moment would set a scale and lead to gaussian behaviour. The scale-invariant random walk is equivalent to studying the addition of random variables with infinite moments. Early examples of scale invariance were treated as paradoxes (see Box 1), but the general mathematics for determining probability distributions for the addition of random variables with infinite moments were developed by Paul Lévy in the 1920s and 1930s. Lévy's study of random processes with $\langle R^2 \rangle = \infty$ did not readily find physical applications because measurements of time-dependent relationships were generally required, of the form

$$\langle R^2(t) \rangle \sim t^\gamma \quad (2)$$

where t is time and γ is a constant. Lévy's ideas at first did not seem to address such problems, as time does not enter explicitly into the original Lévy process. To make the connection, one must take into account the time to complete a jump of the random walk. Strange kinetics for chaotic hamiltonian systems fall outside the domain of brownian motion, and their statistical nature is reflected by values of γ between unity (brownian motion) and two (ballistic motion). Turbulent diffusion, in an open system where energy is pumped in through mixing, is characterized by the value of $\gamma = 3$.

Here we explore stochasticity and dynamics leading to equation (2) and tie both worlds together through the common thread of

BOX 1 The St Petersburg paradox

This classic paradox provides us with a beautiful example of a kind of scaling. The problem involves a game of chance. The game is to flip a coin until a head appears. There is a probability of $\frac{1}{2}$ that this occurs on the first flip, and a probability of $1/2^n$ that the first head appears on the n th flip. Suppose you win 2^n coins if $n-1$ tails occur before the first head. Then your expected winnings are $(1 \times \frac{1}{2}) + (2 \times \frac{1}{4}) + \dots + (2^n/2^{n+1}) + \dots = \infty$. This game was introduced by Nicolaas Bernoulli (the nephew of Jacob and John) in the early 1700s, and is known as the St Petersburg paradox because Daniel Bernoulli wrote about it in the Commentary of the St Petersburg Academy. The question is how many coins a player has to risk (the ante) to play. Ideally, in a fair game, the ante should be equal to the expected winnings. The banker requires the player to ante an infinite number of coins because this is his expected loss. The player, however, favours a small ante because he will only win one coin half of the time, two coins or fewer with probability $\frac{3}{4}$, four coins or fewer with probability $\frac{7}{8}$, and so on. The two parties cannot come to an agreement because they are trying to determine a characteristic scale from a distribution which does not possess one. An infinite number of possible scales of winning (in powers of two) enter, but no scale is dominant. The discovery of this paradox was to cast doubt on the firm mathematical foundations of probability theory. Today, we see this paradox as a rich example of scaling with all its inherent exponents, fractal dimensions, and renormalization scaling properties.

fractal trajectories. First we discuss briefly the problem of a kinetic description of dynamical systems with chaotic behaviour, and mention some important topological properties of the phase portrait of the system. Then, within the context of the history of probability theory, we describe random processes (Lévy flights and Lévy walks) that are relevant to dynamical systems whose orbits possess fractal properties. We consider several examples of dynamical systems with 'symptoms' of Lévy-like processes. Finally we present a phenomenology of a kinetic description of dynamical systems undergoing chaotic motion ('strange kinetics') with fractal properties.

From chaotic dynamics to strange kinetics

An early step to describe kinetics in hamiltonian systems was the introduction of the Fokker-Planck-Kolmogorov (FPK) equation (or its equivalent). It seemed suitable as a natural tool for the description of the slow evolution of system variables¹. The reason for this can be qualitatively explained by considering the 'standard map' of Chirikov-Taylor². This map is obtained when considering the periodically kicked rotor

$$p_{n+1} = p_n + K_0 \sin x_n, \quad x_{n+1} = x_n + p_{n+1} \quad (3)$$

where p and x are rotational momentum and phase of the rotor and n corresponds to the n -th instance of a kick, so n plays a role of discrete time. After each kick the rotor's momentum changes by $\Delta p = p_{n+1} - p_n$, which is proportional to the amplitude K_0 , of the kick, and the changing of phase $\Delta x = x_{n+1} - x_n$. The map of equation (3) is typical of many physical problems and models many features of the occurrence of chaos. If $K_0 \gg 1$, then phase x , taken always in the interval $(0, 2\pi)$, changes randomly. Averaging over the phase, one can get easily from equation (3) the moments $\langle \Delta p \rangle = 0$, $\langle (\Delta p)^2 \rangle = K_0^2/2$ where $\langle \cdot \rangle$ means averaging over x in the interval $(0, 2\pi)$. These simple expressions lead eventually to the diffusion (FPK) equation

$$\frac{\partial F(p)}{\partial t} = \frac{1}{2} D \frac{\partial^2 F(p)}{\partial p^2}, \quad D = \langle (\Delta p)^2 \rangle = K_0^2/2 \quad (4)$$

which describes the slow evolution of the momentum distribution function $F(p)$. This is the simplest manner in which a kinetic description arises in a dynamical system with chaotic behaviour. It is due to the randomness of the fast variable phase, generated by nonrandom equations such as (3) above.

Realistic physical systems appear to be more peculiar because of the inhomogeneity of their phase space where regions of both chaotic dynamics and regular (or quasiperiodic) dynamics are present. This phenomenon is often referred to as a 'stochastic sea' with 'islands' of stable nonrandom orbits (see Fig. 1 with explanations). The existence of such islands and their borders alters the pattern of diffusion dynamics, and early studies led

to a different expression from that of equation (4) for the diffusion coefficient D (ref. 3).

$$D = K_0^2/2\{1 - 2J_2(K_0)\}$$

where J_2 is a Bessel function. The cause of the change in the $D(K_0)$ dependence is more important than the change itself: it arose from the discovery of a new topological object in phase space⁴⁻⁶ which Percival called a cantorus. A cantorus can be imagined as a closed curve except for an infinite number of gaps, immersed in the stochastic sea. The gaps have different sizes so that the measure of the cantorus is zero (therefore a cantorus is fractal, rather than a typical curve which has the measure one). All points of a cantorus belong to the only orbit if the initial condition has been chosen from the cantorus manifold. Motion along cantori is periodic and unstable. Every island or set of islands (see Fig. 1) is enclosed with an infinite set of cantori.

The discovery of cantori changed the understanding of phase space structure and the transport within it. Particle transport takes place within the area of the stochastic sea and corresponds to a wandering amidst the islands. The wandering particle can pass through the gaps of cantori, which creates effective barriers for diffusion. The cantori closest to the island boundaries seem to be the most important, because their barriers are the widest and their gaps are the narrowest. The passage through the intervals between the islands is complicated by the fact that the nearest cantorus blocks the escape of the particle from the boundary layer in the vicinity of the island. The particle gets trapped inside the area between the island border and the nearest cantorus (or cantori) and this is the reason why one can observe higher densities of dots in the stochastic sea of Fig. 1. We cannot see cantori in Fig. 1, but they disclose themselves as boundaries of dark strips inside the stochastic sea domain.

This tendency to get trapped has been called 'stickiness', and it is the existence of 'sticky' orbits that makes the kinetics of the particle strange. Let us describe this in more detail. Consider a particle wandering in the stochastic sea area which passes through a gap of a cantorus very close to an island boundary. The particle now experiences a new kind of motion and almost regularly rotates around the island or around a set of islands. The rate of instability (Lyapunov exponent) near the island's boundary is small, so the motion is close to a quasiperiodic one. In addition, this cantorus (or cantori) blocks the possibility of escaping from the boundary layer area. As a whole it works as a trap for the particle. Thus trapping of the particle in the (x, p) plane corresponds to a 'flight' along the curve A_1A_5 in Fig. 1. In the multidimensional case, flight can be perpendicular to the (x, p) plane direction. In other words, trappings and flights are complementary features of anomalous excursions of the

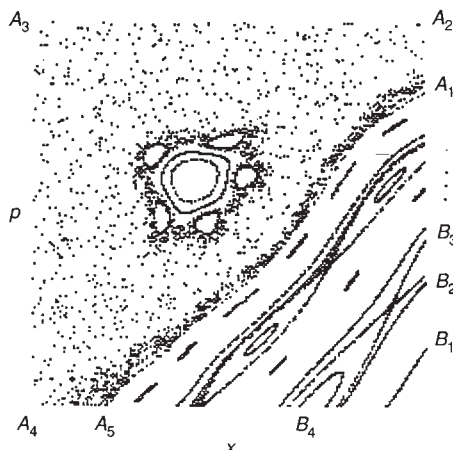


FIG. 1 Illustration of the topology of dynamical system orbits in the phase space (x, p) . Starting from some initial conditions, the orbit is generated by an infinite set of iterations, equation (2), and each iteration gives a dot on the (x, p) plane. Such a procedure is called a Poincaré section. Instead of a continuous curve, the orbit is represented on the (x, p) plane by the set of values (x_n, p_n) taken at special set of time instants. We plot a typical part of the (x, p) plane for equation (2) with $K_0=1.2$. The regular orbits ($B_1, B_2, B_3, B_4, \dots$) describe quasiperiodic motion. In contrast to them, there is an orbit which fills the domain $A \equiv (A_1, A_2, A_3, A_4, A_5)$ (except for some area in its central part). There is no possibility of fitting all of these points onto one curve. This orbit represents chaotic motion. The area A is a 'stochastic sea'. Inside A there is a set of 'islands' in which the motion is regular. Domain A borders a big island along the curve A_5A_1 (there is only a part of the island in the figure and the orbits $B_1, B_2, \dots$ are closed in this island). The density of points in the 'stochastic sea' area (or distribution function) displays how often this part of the phase space has been visited. The density is inhomogeneous and the most visited parts are narrow strips close to island boundaries. Dark strips of high density points are distributed along the border (A_1A_5) of the big island, around the smaller island and its five satellite sub-islands.

random-walk particle orbit. Such behaviour is not new in physics. In the theory of turbulence it is known as intermittency, and there are many examples of intermittent behaviour of dissipative dynamical system⁷, as well as of hamiltonian systems⁸. Actually the analogy with turbulence may be even deeper as will be seen later.

The crucial questions are what the probabilities are of anomalous orbits with strong intermittent dynamics, or how often one chaotic orbit will happen to exhibit intermittent bursts. It seems that the ability of dynamical chaos to have anomalous statistical properties is general, although sometimes it is difficult to observe. There are many examples of anomalous kinetics of particles due to flights and trappings: simulations of passive scalar dynamics in fluids⁹⁻¹¹, charged particle multidimensional dynamics¹², particle dynamics in two-dimensional periodical and quasiperiodical potentials^{13,14}, and experiments on transport in the presence of convective cells¹⁵ or in the presence of regular patterned capillary waves¹⁶. Correspondingly, for flight observations there is evidence of anomalous diffusion of particles, so that in the expression for particle displacement R after time t (averaged over a number of orbits)

$$\langle |R| \rangle \sim t^\mu \quad (t \leftarrow \infty) \quad (5)$$

the exponent $\mu \neq \frac{1}{2}$, which would be the value for normal diffusion (we are using the form of equation (5) instead of equation (2) for convenience). Sometimes μ can be close to one, corresponding to enormously strong enhancement of the transport ('ballistic motion'). Applications of this phenomenon include plasma fusion and understanding the spread of environmental pollution.

It seems now that the 'strange kinetics' described above are similar to the 'Lévy process'¹⁷ which appeared as an alternative to the gaussian law of large numbers or the usual brownian motion. It is worth giving this story separately because it helps when trying to understand its deep relationship to dynamical chaos.

From St Petersburg to strange kinetics

We have mentioned that the complexity of chaotic orbits near the boundaries of islands resides in their self-similar behaviour on a small scale. To reflect such a property one cannot use a correlation length or a characteristic size. One has to introduce many scales, expecting a self-similarity among all of them. The history of probability theory has already provided us with an example of this type of scaling.

An example of random walk, formulated in the eighteenth century and known now as the St Petersburg paradox, has shown

the existence of infinite moments (Box 1). Lévy generalized¹⁷ the central-limit theorem to take such possibilities into account. He considered the distribution of sums of random variables with infinite moments. Let us add up several identically distributed random variables X_i . The value of each variable X_i can be thought of as a step in a random walk. Each jump length is chosen from a distribution $p(x)$. Lévy asked when the distribution of the sum of n steps $p_n(x)$ (up to some scale factors) would be the same as the distribution of any term in the sum, $p(x)$. This is basically the question of fractals: when does the whole (the sum) look like one of its parts? One answer to this question is well known: a sum of gaussians is a gaussian. The distribution of the X_i is $p(x) = (2\pi)^{-1/2} \exp(-x^2/2)$, the distribution of $p_n(x)$, the properly scaled sum $(n)^{-1/2} \sum_i X_i$ is $(2\pi n)^{-1/2} \exp(-x^2/2n)$. So $p(x)$ and $p_n(x)$ have the same distribution up the scale factor n . In Fourier space ($x \rightarrow k$) $p_n(k)$ has a simple form

$$p_n(k) = \int_{-\infty}^{\infty} p_n(x) \exp(ikx) dx = \exp(-nk^2/2) \quad (6)$$

Note that the second moment of $p_n(x)$ is given by $-\partial^2 p_n(k=0)/\partial k^2 = n$. Lévy discovered that other solutions existed such that $p_n(x)$ and $p(x)$ had the same distribution. He found this to be the case when

$$p_n(k) = \exp(-\text{constant} \times n|k|^\alpha) \quad (\text{for } 0 < \alpha \leq 2) \quad (7)$$

The $\alpha = 2$ case is the gaussian which we have just studied. For $\alpha < 2$, we note that $\langle x^2 \rangle = -\partial^2 p_n(k=0)/\partial k^2$ is infinite. These random walks with steps with infinite second moments are known as Lévy flights. It now seems obvious that to have scale-invariant distributions, we would need to sum up random variables with no scale. Indeed, applying an inverse Fourier transform to equation (7) gives

$$p_n(x) \sim \text{constant} \times n/x^{1+\alpha} \quad (8)$$

BOX 2 Weierstrass random walks

To obtain a deeper understanding of the new kind of the characteristic function $p(k)$ in equation (7) in the text, consider a special example of random walk which can be written in the form

$$p_w(x) = \frac{\alpha-1}{2a} \sum_{j=0}^{\infty} a^{-j} (\delta_{x+b^j} + \delta_{x-b^j}) \quad (*)$$

where δ_{xy} means the Kronecker symbol. Jumps of size $\pm 1, \pm b, \pm b^2$ and so on can occur, but jumps an order of magnitude longer in base b occur an order of magnitude less often in base a . We make about a jumps of length unity before, on the average, a jump of length b occurs, and so on, until in a hierarchical fashion patchy clusters of all sizes are formed. Taking the Fourier transform of $p_w(x)$, we arrive at

$$p_w(k) = \frac{a-1}{a} \sum_{j=0}^{\infty} a^{-j} \cos(b^j k)$$

which is the Weierstrass function. The important part of this result is that the random walk process (*) describes a situation that reflects the scaling of the St Petersburg paradox. This process is related to the Weierstrass function which possesses the scaling property:

$$p_w(k) = a^{-1} p_w(bk) + \left[\frac{a-1}{a} \right] \cos(k)$$

The detailed analysis of this equation^{19,43} gives the final result in the form

$$p_w(k) \sim 1 - |k|^\alpha \exp(-|k|^\alpha)$$

with $\alpha = \ln a / \ln b$ when $b^2 > a$ so $\langle x^2 \rangle$ is infinite. Value α represents the fractal dimension of a random walk path^{18,19,43}. This exponential form with the fractional power comprises the non-gaussian solution to Lévy's question addressed in equation (7). If one wants to add random variables (take a random walk) and have the probability distribution after n steps look like the probability distribution after one step (except for a change of scale), then your random variables are either gaussian or have infinite second moments. This means the solution is either gaussian or fractal. The process $p_w(x)$ is used to illustrate Lévy flights in Fig. 2.

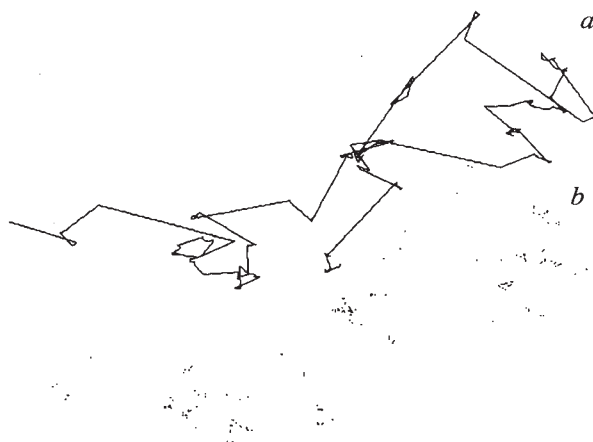


FIG. 2 The connected lines a show the trajectory of a Lévy walk. The isolated dots b show the points visited by a Lévy flight which represents the turning points of the Lévy walk in a . The trajectory is plotted from a two-dimensional version of equation (*) in Box 2 with $\alpha = \ln 2 / \ln 3$. Number of points is 350.

where the power law for the tail of the distribution indicates the absence of a characteristic size unlike the gaussian distribution, where $\alpha = 2$. We emphasize that sums governed by equation (8) with $\alpha < 2$ are dominated by their largest terms, and thus by rare intermittent events. It can also be seen from equation (8) that the power-law behaviour of the tail of the distribution function defines the appreciable probability of large values of displacement x , explaining the reason for 'Lévy flights'. The exponent α will turn out to be the dimension of the point set visited by a Lévy flight. For Lévy flights this dimension is fractal ($0 < \alpha < 2$). Mandelbrot has connected the fractal properties of random walks with Lévy flights¹⁸ (see also ref. 19). This may be important especially for turbulence problems, discussed below.

A non-poissonian distribution is the forerunner of the concept of fractal time where the waiting times between jumps occur on all scales, but with order of magnitude longer waits occurring an order of magnitude less often. For fractal time the average, $\langle t \rangle$, between events is infinite. This does not mean that the duration between every event is infinite, just as in the coin game of the St Petersburg paradox not every player wins an infinite amount of money just because the expected winning is infinite.

It is not so difficult now to introduce fractal time^{20,21} into random walks. In some sense it could be done in a similar manner to the fractal space random walk. Indeed, let the time sequence $t_0, t_1, \dots \equiv \{t_j\}$ be the sequence of moments when steps of the random walk are performed. Formally there are no restrictions on the structure of the $\{t_j\}$, especially in a dynamical

system and $\{t_j\}$ is (for example) a set of crossing times of the Poincaré section. A more detailed description is based on the introduction of a time delay function $\psi(t)$ which is the probability density that the duration between events is t . The behaviour of $\psi(t)$ may be similar to the scaling of the Weierstrass function (Box 2). As will be seen later, the combination of fractal time and space for random walks can create essentially a new kind of wandering which is the background of strange kinetics.

Lévy flights and walks

Our discussion has so far evolved from the observation that chaotic diffusion in dynamical systems can be shown by particle trajectories which are reminiscent of Lévy flights. (Evidence of this will be given in the next section). Beautiful and elegant as the mathematics of Lévy flights are, they are not directly applicable to the kinetic description of real dynamical processes. One must take into account the time to complete each jump of the random walk. This process we have named the Lévy walk²². One then looks at the displacement after a time t rather than an n steps. Even when the average jump distance is infinite (a Lévy flight) the average displacement after a time t (a Lévy walk) will be characterized by a time-dependent growth, such as t^μ in equation (5). Lévy walks are a modification of the flights, preserving the spatial self-similarity. To overcome the divergence in $\langle R^2 \rangle$, a time cost is introduced so that long steps are penalized. The formulation of Lévy walks rests on a continuous time random walk (CTRW) approach. The nature of the walks is entirely specified by the probability distribution $\Psi(r, t)$ for a single step of r in time t . This probability density has the following property.

$$\int dr dt \Psi(r, t) = 1, \quad \Psi(r, t) = p(r) \delta(t - |r|/v(r)), \quad (9)$$

$$p(r) \sim |r|^{-\mu} \quad r \rightarrow \infty$$

The second property relates the step length to time in terms of a length-dependent velocity. This space-time coupling is mathematically essential to avoid the divergences of Lévy flights. The third property is responsible for the self-similarity of trajectories and by itself represents the Lévy flight. The 'secret' in getting rid of the divergence typical of the flight's mean-square displacement lies in the space-time coupling of $\Psi(r, t)$ through the δ -function. Space-time fractal behaviour is introduced directly through coupling on $\Psi(r, t)$. Figure 2 shows a two-dimensional realization of a Lévy walk where $v(r) = \text{constant}$ and the fractal dimension is $\ln 2 / \ln 3$. The self-similar aspect of the picture is important: a series of small steps is followed by larger ones, which are then followed by larger ones still; furthermore, no particular length scale dominates. These features of the Lévy walk make them attractive in simulating momentum mixing over large distances, and they are therefore candidates for turbulent diffusion and turbulent flows²³. In fact, Hayot²⁴ was able to take advantage of this picture and calculate the velocity profile for inertial range turbulent pipe flow, and turbulent flow past a cylinder.

Phase rotation in Josephson junctions provides an example of a Lévy walk. For a constant voltage across a junction, the phase difference between current and voltage will rotate at a constant rate. Under certain conditions, however, involving the amplitude of a driving force, the phase can switch back and forth intermittently between clockwise and counterclockwise rotations²⁵⁻²⁷. One can consider the distribution of times spent in a given rotation state. If the phase rotates n times in the clockwise sense we consider this as a step of length n to the right in a one-dimensional random walk. The junction can be operated such that the distribution $p(n)$ for having n consecutive rotations in the same direction will be a Lévy distribution possessing an infinite second moment. But the mean value of $\langle x^2(t) \rangle$, where x is the phase difference, will not be infinite. This

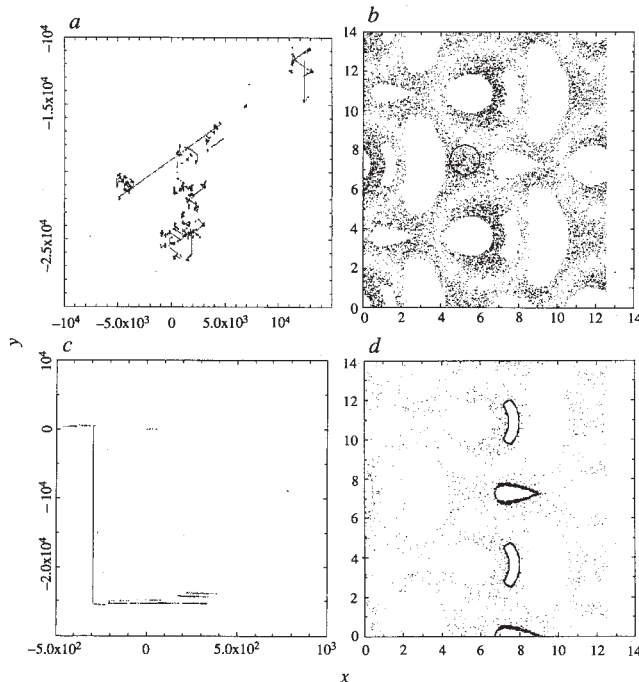


FIG. 3 Poincaré section of a streamline (or passive particle) with $\varepsilon = 2.3$ (ref. 42). Each dot represents a crossing of the streamline (or particle orbit) with the plane $z = 0 \pmod{2\pi}$. This corresponds to anomalous transport with $\mu > \frac{1}{2}$ (see Fig. 4). a, A general impression of the particle's wandering. There are many tracks which represent flights. Some of them have length of order 10^4 . b, Small-scale plot of the same orbit. The area is of order $4\pi \times 4\pi$. They are a part of the stochastic web and many islands. The full stochastic web can be obtained from b by its periodic continuation in x and y directions. c, A special part of the orbit which corresponds to an initial condition taken inside the circle in b. It is a very long flight of order of 10^4 . If we plot all the points c onto the small square $x \pmod{4\pi}$, $y \pmod{4\pi}$ then we obtain a new visualization, d. Dark strips around some islands correspond to frequent visits to these areas by the particle; they result from trapping due to the stickiness of the islands' boundaries. This picture confirms that islands create the tracks for flights.

is because each rotation takes a finite time to be completed, so by time t only a finite number of rotations can occur. The scaling of the mean squared displacement $\langle x^2(t) \rangle$ is a function of time and can exhibit the accelerated behaviour $\langle x^2(t) \rangle = t^\mu$ with $1 < \mu \leq 2$ (ref. 26).

Even more accelerated motion is possible and the law $\langle R^2(t) \rangle \sim t^3$ is well known for turbulent diffusion, as Richardson's law. For the random walk corresponding to the Josephson junction example, flight paths were traversed with a constant velocity. A Lévy set of trajectories where the velocity depends on the flight length will reproduce Richardson's law²² when the $v(r)$ in equation (10) is given by $v(r) = |r|^{1/3}$, and $p(r)$ describes a Lévy flight. This $v(r)$ relation is the signature of Kolmogorov's scaling for inertial range of turbulence. It represents particle trajectories encountering a hierarchy of larger and larger vortices as time progresses. The Lévy walk approach not only arrives at the proper scaling law, but in addition gives a Lagrangian description of particle trajectories. Modifications to Richardson's law have also been investigated in the framework of random walks^{22,23}.

Patterns, stochastic webs and strange kinetics

We now discuss several dynamical models for which the phenomena of anomalous, non-gaussian transport (see equation (5)) with $\mu \neq \frac{1}{2}$ have been observed. The models describe hamiltonian systems for which the Liouville theorem (of the preservation of phase volume) is relevant. This means that the initial area in the phase, bounded by a surface, can change its form drastically, but the volume surrounded by the surface cannot change. The crucial feature of this treatment is to show how the existence of flights (or trappings) and anomalous kinetics is connected to symmetry properties of a system or, more generally speaking, to a symmetry of the system's phase portrait in phase space²⁵⁻²⁹.

As the first example let us consider the 'Q-flow'²⁸ defined as

$$\mathbf{v} = (\partial\psi_q/\partial y + \varepsilon \sin z, -\partial\psi_q/\partial x + \varepsilon \cos z, \psi_q) \quad (10)$$

where ε is a parameter. Here the generating symmetry function ψ_q is

$$\psi_q = \sum_{j=1}^q \cos(\mathbf{R} \cdot \mathbf{e}_j), \quad \mathbf{R} = (x, y) \quad (11)$$

$$\mathbf{e}_j = (\cos 2\pi j/q, \sin 2\pi j/q) \quad (j = 1, \dots, q)$$

where q is the order of symmetry, and $\mathbf{e}_j$ is set of unit vectors, which form the regular star. Lines of constant ψ_q generate the

q -fold symmetry pattern in the (x, y) plane, which is of crystal-like symmetry for $q \in q_c = \{1, 2, 3, 4, 6\}$ and is of quasicrystal-like symmetry for $q \notin q_c$. The Q-flow is incompressible ($\text{div } \mathbf{v} = 0$) and of Beltrami type ($\mathbf{v} = \text{curl } \mathbf{v}$), described in detail in ref. 29. The best known is the case of cubic symmetry flow when $q = 4$ and $\psi_4 = 2(\cos x + \cos y)$, the 'Arnold-Beltrami-Childress' (ABC) flow. Interest in the ABC flow has been raised by the nontrivial chaotic behaviour of the flow's streamlines³⁰⁻³² and by applications to the fast dynamo problem. It should be mentioned that any Q-flow has a stochastic web in real coordinate space^{8,29}, which means the following. The set of equations $dx/v_x = dy/v_y = dz/v_z$ defines the flow's streamlines. Its solution for v from equation (10) displays an infinite connected net of channels of finite width of order ε , inside which streamlines look like random contours, which leads one to conclude that passive scalar dynamics is diffusive. We will see later that the transport through the stochastic web is anomalous or 'strange' for general situations^{9,10}, implying the existence of flights and trappings, and also implying that $\mu \neq \frac{1}{2}$ in the asymptotic formula, equation (5).

Observations of flights for different models can be sketched as in Fig. 3. Flights (or trappings) appear along the island's boundary, and the existence of pattern symmetries is crucial for determining the probability of their occurrence. If the patterns are destroyed, the long flights and anomalous transport properties disappear. This is why the generation function ψ_q in equation (10) is of great importance. It possesses general symmetry properties and creates different directions of flights in the phase space and real space of Q-flow. In particular, changing the parameter ε in equation (10) changes some aspects of the phase portrait. The number of islands, their locations, sizes and shape all depend on ε . The levels of stickiness of the island boundary, and the cantori closest to them, are also functions of ε . This is revealed in the sensitive dependence of μ on ε . For $q = 6$ (hexagonal symmetric flow) the curve $\mu = \mu(\varepsilon)$ was obtained in ref. 9. (See Fig. 4) All variations of μ in the plot correlate one-to-one with sharp changes in the phase portrait of the system after ε has passed through some critical values. The influence of the closeness of ε to such a critical value was found¹⁰ for the equations (10 and 11) with $q = 5$. Figure 5 explains how this could happen. For some intervals of ε there are special domains in the phase space of very long particle trapping which creates extremely long flights, called stochastic jets. The escape of any orbit from a bundle of orbits, initially inside the trapped chaotic area, is blocked by a cantorus. The scattering of the bundle is very small and the bundle survives as a long jet. The time of

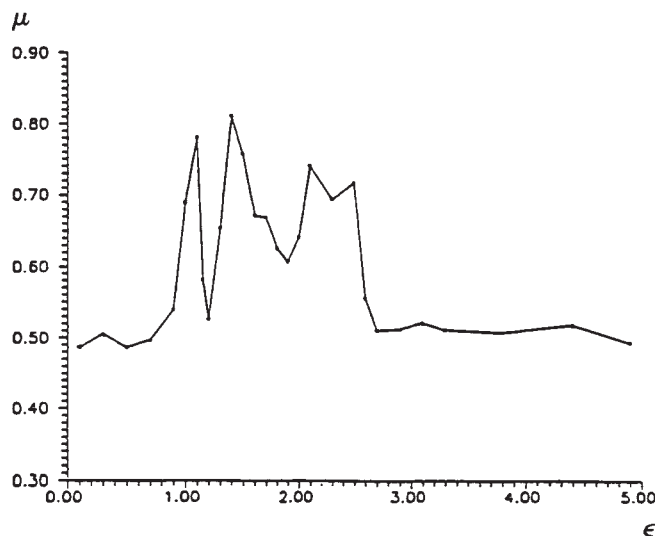


FIG. 4 The dependence of the diffusional exponent $\mu = \mu(\varepsilon)$ (ref. 9), for the same hexagonal flow as in Fig. 3. There is a region of ε between ~ 0.8 and 2.8 where μ fluctuates strongly between $\frac{1}{2}$ and 1 .

survival of a jet can be comparable with any timescale of observation or simulation. Such jets imply that the exponent μ is close to one (ballistic behaviour).

Another model, for which flights, stochastic jets and anomalous transport were observed in detail¹² is the four-dimensional mapping

$$\begin{aligned} u' &= [u + K \sin(v - z)] \cos \alpha_0 + v \sin \alpha_0 \\ v' &= -[u + K \sin(v - z)] \sin \alpha_0 + v \cos \alpha_0 \\ p' &= p + K_0 \sin(v - z), \quad z' = z + p' \end{aligned} \quad (12)$$

Equations (12) describe particle motion in a magnetic field and in a wave packet which propagates obliquely to the magnetic field. Here u is a dimensionless velocity along x and v is the coordinate x ; p and z are dimensionless velocity and coordinate along the magnetic field; α and K, K_0 are dimensionless parameters. The first two equations in (12) describe the so-called web-map which generates the stochastic web of the q -fold symmetry if $\alpha_0 = 2\pi/q$ with integer q . Averaging the hamiltonian for the first two equations in (12) will coincide with the pattern generator ψ_q in equation (11) if one puts $\vec{R} = (u, v)$. The second pair of equations in (12) coincides with the standard map, equation (3). Thus system (12) described coupling of the standard and web maps. It demonstrates a rich collection of different dynamical features. Even for small K and K_0 the diffusion can

be fast and anomalous. This diffusion is faster than the Arnold diffusion. Figure 6 represents the two cases of diffusion when trappings and flights occur after a fairly small parameter change. As in previous cases, the system in equation (12) possesses a symmetry in its phase space, which generates flights of different forms.

Clusters, multifractals and different length scales

An important observation¹² was the dependence of the diffusion exponent μ on the phase-space region where the set of initial coordinates is taken. This property reflects the inhomogeneity of μ for the same set of parameters (K, K_0, α). It is known that turbulent motion can be characterized by such inhomogeneous

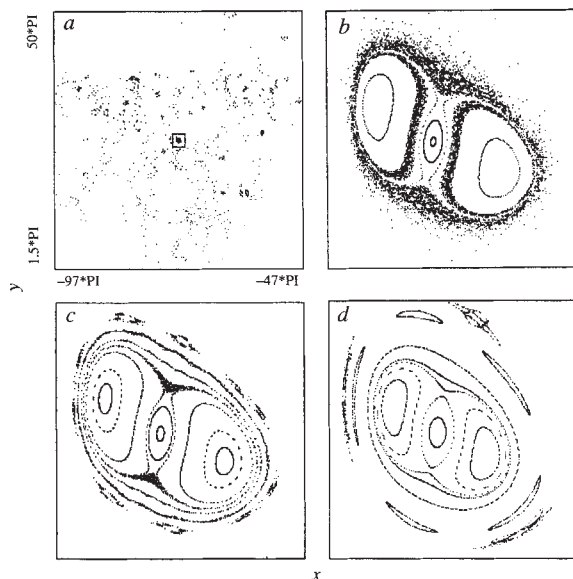


FIG. 5 The topological situation in phase space when extremely long flights, or trappings, occur¹⁰. We call them stochastic jets. All plots correspond to the 5-fold symmetry Q -flow ($q=5$ in equations (16) and (17)). Each plot is the (x, y) -coordinate of the only streamline when its z -coordinate value is $z=0 \pmod{2\pi}$. The case a plots long time transport for $\varepsilon=0.8$. Many black clusters correspond to the streamline trapping in a small domain. Zooming into the small square in a gives b , which shows a figure '8'-like region with chaotic behaviour outside and inside the 8. These 'outer' and 'inner' areas of chaos are slightly connected (the white narrow strip does not absolutely separate them). This reflects small probability of penetration from the 'inner' chaotic area to the 'outer' one. If a particle has been trapped in the 'inner' area, it stays for a very long time which results in an appearance of jets in the perpendicular z -direction. Small variations of the parameter ε change the phase portrait of the system (c and d). In c , $\varepsilon=0.5$. A closed curve exists between 'inner' and narrow chaotic area and 'outer' one and the stochastic jets like in b have disappeared. The same is observed in d , where $\varepsilon=0.4$, and the 'inner' chaotic area is invisible. The existence or nonexistence of a low probability connection between 'inner' and 'outer' domains in $b-d$ means that a wandering (outside the figure '8') particle can either be trapped for a long time in a small domain or cannot be trapped. This provides a micro-picture of the occurrence of superlong flights and trappings.

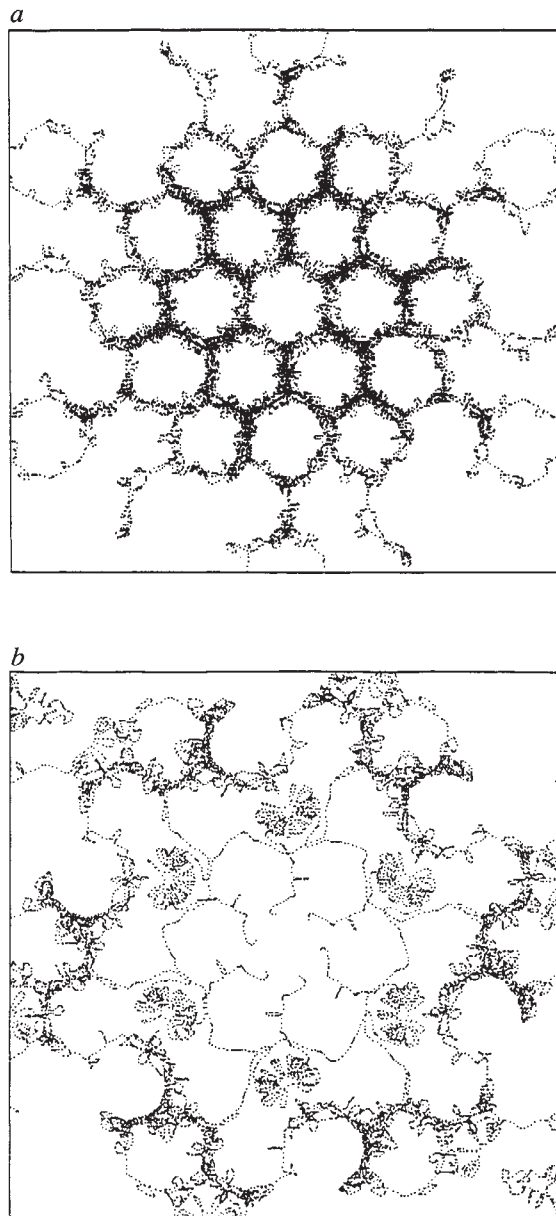


FIG. 6 The trajectory in a and b is part of a Lévy flight which does not possess any characteristic scale. If each segment is followed with a velocity proportional to the cube root of the segment length, then Richardson's law of turbulent diffusion ensues. Such trajectories can be used to simulate high Reynolds number fluid flows and are projections of particle's orbit onto the (u, v) plane for $\alpha_0=2\pi/6$ (hexagonal symmetry and $K=0.18$, for the model described by equations (18). In a , $K_0/K=0.3$ and diffusion through the stochastic web is close to the normal one. In b , $K_0/K=0.08$ and there are many flights along the web.

fractal properties³³. One can imagine a fractal net or cluster imbedded into the real space, along which diffusion can occur. Now imagine several different nets or clusters imbedded into the same space. This provides a simplified representation of a multifractal: complicated, interpenetrating network of different fractals. In some sense, the same kind of idea is applicable to dynamical chaos. One can suppose the existence of local fractal dimensions in the phase space and clusters of different dimensionality. A particle diffusing along a cluster can switch from time to time to another cluster. If the measure of clusters with long flights is considerable, then their input to the diffusional exponent μ should be important. This, along with finite observation times and the choice of initial conditions, will define the final value μ

$$\mu = \mathcal{M}(\alpha, \beta; L, T) \quad (13)$$

where α and β are fractal exponents of local space and time singularities of the transition probability (see below); L and T are large-scale distance and time, and $\mathcal{M}$ is the corresponding functional. Let us mention that the usual definition of the diffusion constant from equation (3)

$$D = \lim_{\Delta t \rightarrow 0, \Delta x \rightarrow 0} \langle (\Delta x)^2 \rangle / \Delta t = \text{constant} \quad (14)$$

gives the necessary connection (equation (13)) in the gaussian case ($\alpha = 1, \beta = 1$) for μ to be equal to $\beta/2\alpha = \frac{1}{2}$.

The cases of the kinetic descriptions in which the processes of flights and trappings are important have been broadly discussed²², especially in the context of fractal space and fractal time. The generalization to the multifractal case is detailed in ref. 12, and the fractional Fokker-Planck-Kolmogorov (FFPK) equation was derived in ref. 34. It is beyond the scope of this article to discuss the FFPK equation and we present here only some of its features. One further consideration is to generalize the idea of diffusion constant. The basic idea involves performing the limit $\Delta t \rightarrow 0, \Delta x \rightarrow 0$ in equation (14). For homogeneous and smooth t and x processes, the procedure is straightforward. When this is not the case, the limit has many flights and trappings. On many scales we encounter the limit (see for example refs 35, 43)

$$\mathcal{B} = \lim_{\Delta t \rightarrow 0, \Delta x \rightarrow 0} |\Delta x|^{2\alpha} / |\Delta t|^\beta = \text{constant} \quad (15)$$

which replaces equation (14) and produces the scaling $x^2 \sim t^{\beta/\alpha}$. It is not a simple matter to obtain the unknown constants α and β from the properties of the phase space of a system because they should come from the analysis of fine details of the cantori described earlier. Nevertheless one can assume that invisible cantori are in charge of the local properties of the phase space and the limit (equation (15)) defines the large-scale properties of the transport process. It has been shown^{12,34} that the constant μ in equations (5) and (13) is $\mu = \beta/2\alpha$, and that is the way in which fractal properties of space and time (fractal because α and β in equation (15) are fractional) allow us to deduce anomalous transport, connecting fractal space and time inherent in Lévy-like processes¹⁹.

The above examples show that chaotic dynamics are related to the phenomena of Lévy flights and walks. One should expect, however, that different intermediate asymptotics could exist which correspond to normal diffusion as well as to anomalous transport with different exponents. Such a picture is consistent with the general multifractal property of chaos.

Scaling and strange kinetics have many applications to situations other than those discussed here: the physics of fluids, plasmas, macromolecules, computer sciences, chemical kinetics and technological problems^{23,24,36-41}. The potential for strange kinetics exists in any situation where some hierarchical ordering of the random walk occurs. It happens in the random walk of electrons in disordered media (when localized states are of importance^{21,36,40}), in the kinetics of macromolecules and in aggregation processes where a long-range correlation exists^{38,39}. Problems of anomalous diffusion and anomalous conductivity in plasmas provide a good number of examples of kinetics with scaling³⁷. We hope that a unified understanding of these examples could build a generalized approach for a kinetic theory of processes with long-time memory and long-range correlations as now exists for gaussian-like random dynamics. □

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Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain

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The three-dimensional structure of the basic/helix-loop-helix/leucine zipper domain of the transcription factor Max complexed with DNA has been determined by X-ray crystallography at 2.9 Å resolution. Max binds as a dimer to its recognition sequence CACGTG by direct contacts between the α -helical basic region and the major groove. This symmetric homodimer, a new protein fold, is a parallel, left-handed, four-helix bundle, with each monomer containing two α -helical segments separated by a loop. The two α -helical segments are composed of the basic region plus helix 1 and helix 2 plus the leucine repeat, respectively. As in GCN4, the leucine repeat forms a parallel coiled coil.

THE helix-loop-helix (HLH) transcription factors are characterized by a highly conserved bipartite DNA-binding region¹. The portion responsible for dimerization of these proteins consists of two amphipathic α -helices connected by a loop², hence the name HLH. Immediately N-terminal to the HLH region is a conserved basic region (b) which is required for, and probably mediates, DNA binding by HLH dimers³⁻⁸. Family members occur in diverse eukaryotes ranging from mammals to yeast, and are important in regulating metabolism, cell differentiation and development (Fig. 1a; ref. 1 for a review). The family includes some proteins with no basic region, which do not bind DNA, and act as negative regulators of the HLH transcription factors that do contain the basic region^{9,10}.

The Myc oncoproteins contain well conserved b and HLH motifs, followed by a conserved heptad leucine-repeat or zipper (Z) motif (see refs 11 and 12 for reviews). As for the b/HLH proteins, the biological activities of Myc are predicated on DNA binding, which depends on the entire b/HLH/Z region. Sequence comparisons indicate that this b/HLH/Z motif occurs in a large number of biologically important proteins (see Fig. 1a for a partial sequence alignment). These molecules form a variety of homo- and hetero-oligomers, resulting in multiprotein complexes with diverse DNA-binding properties and biological effects.

Max is a b/HLH/Z protein that hetero-oligomerizes with the Myc oncoproteins, enabling them to bind DNA under physiological conditions^{13,14}. *In vivo* Max is associated with Myc^{15,16}, and is required for malignant transformation by Myc¹⁷⁻¹⁹. Unlike Myc, which does not normally homo-oligomerize or bind DNA, Max readily homodimerizes and binds DNA with high affinity^{14,20-22}. Characterization of other b/HLH/Z proteins interacting preferentially with Max^{23,24} indicates that Max may be central in orchestrating the biological activities of b/HLH/Z transcription factors.

We now report the X-ray crystallographic structure determination of a canonical b/HLH/Z protein bound to its DNA-recognition element. The three-dimensional structure of the DNA-binding domain of the Max transcription factor bound to its cognate DNA, obtained at 2.9 Å resolution, demonstrates that Max dimerizes to give a novel, parallel, left-handed, four-helix bundle structure. This structure clarifies the nature of the pro-

tein-protein and protein-nucleic acid interactions that allow Max to dimerize and bind DNA. Moreover, because of the high degree of structural conservation present in the DNA-binding regions within and between the b/HLH/Z and HLH proteins (Fig. 1a), it serves as a three-dimensional scaffold with which to understand DNA recognition and homo- and heterodimerization in these two important transcription factor families.

Structure determination and refinement

A truncated form of Max (Max 22-113), containing the basic, helix-loop-helix and leucine-zipper regions, was expressed, purified and co-crystallized with a 22-base-pair (bp) duplex oligonucleotide containing a specific DNA-binding site from the adenovirus major late promoter (E_{USF}; ref. 25) as outlined in Table 1. The hexagonal cocrystals (space group *P*6₂22; *a* = 72.25 Å, *c* = 146.39 Å) diffract isotropically to 3.2 Å in our laboratory and to 2.8 Å using synchrotron X-radiation. The asymmetric unit includes one Max 22-113 polypeptide and only 11 of 22 bp of the quasisymmetric DNA. Consequently, the DNA in the co-crystal is averaged over two orientations, as seen in an unrelated, crystalline DNA²⁶. This 2-fold averaging does not affect the central 6-bp specific recognition element (CACGTG) or the protein, which binds DNA as a symmetric homodimer. Of the 8 bp flanking the central CACGTG, two conform to the dyad symmetry and the other 6 bp do not. Presumably this packing arrangement reflects the fact that co-crystal formation is largely controlled by the symmetry of the specific protein-DNA complex formed by the Max homodimer and the palindromic recognition sequence, CACGTG (see later).

The structure was solved at 3.3 Å resolution by multiple isomorphous replacement (MIR) using five iodinated DNA derivatives (Table 1). The heavy atom site for derivative IdU4R was located by difference Patterson analysis, and its position and occupancy were refined²⁷ to give an initial figure-of-merit (f.o.m.) of 0.36. The remaining four derivatives were characterized by difference Fourier syntheses, and refinement against both isomorphous and anomalous differences gave an overall f.o.m. of 0.58. The 3.3 Å-resolution MIR map revealed clear electron density for the phosphodiester backbone, most of the bases, and a long α -helix corresponding to the basic region plus H1 (b/H1; see legend to Fig. 1a). 'Solvent flattening' with SQUASH²⁸ permitted a canonical B-form DNA with the sequence shown in Fig. 1c to be positioned in the electron density map by overlaying thymine methyl carbon atoms on the

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corresponding iodine atomic coordinates, giving a root-mean-square (r.m.s.) deviation of 0.93 Å. Thereafter, base pairs were manually repositioned²⁹ to fit the modified electron density, and the DNA model was further improved using Powell minimization³⁰. Phase combination³¹ with the MIR data and the refined DNA model revealed clear electron density for the b/H1 α -helix and most of the α -helix corresponding to H2 plus the leucine zipper (H2/Z), which were initially built as two α -helical polyalanine segments. Several rounds of model building, phase combination, and refinement allowed an unambiguous trace and sequence assignment of the polypeptide chain from Ala 22 to Ser 107 (94% of Max 22–113) with an *R*-factor of 27.6%.

The model was then refined at 2.9 Å resolution against data measured with synchrotron X-radiation, using simulated annealing³² and Powell minimization. Omit $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier syntheses were calculated with the refined model, and several rounds of manual rebuilding and positional refinement were carried out to improve

stereochemistry. Finally, tightly restrained, individual isotropic B-factors were refined. The current crystallographic refinement model comprises the DNA 11-mer oligonucleotide depicted in Fig. 1c and the Max 22–113 amino-acid sequence from Ala 22 to Ser 107. No attempt was made to place solvent molecules. Refinement statistics are given in Table 1, and portions of the final electron density are shown in Fig. 2b and c. The electron density for the polypeptide backbone for b/H1, the loop and H2 is everywhere continuous at 1σ in a $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier synthesis. Towards the C terminus of the leucine zipper, where the electron density is not as well defined, there are a few density breaks along the polypeptide backbone. A Ramachandran analysis³³ showed only 1 of 85 backbone torsion angle combinations in an unfavourable region of the (ϕ, ψ) plot (data not shown). Omit maps were used to examine the electron density for the protein, which revealed no evidence of multiple conformations of the polypeptide backbone at this resolution limit. Some of the solvent-exposed side chains naturally had alternative conformations, but no attempt was made to include these in the refinement.

Before describing the structure in detail, the crystallographic implications of partial, 2-fold averaging of the DNA within the co-crystal requires some discussion. There is well defined electron density for the 3 bp adjacent to the 2-fold crystallographic axis relating each half of the dimeric protein–DNA complex. Beyond the confines of the central CACGTG recognition sequence (highlighted in Fig. 2b and c), the electron density for the DNA bases is present, well connected, and consistent with a superposition of two different base pairs in each of the non-palindromic positions. We therefore have to limit our discussion of side-chain–base contacts to the 2-fold symmetric Max recognition sequence CACGTG. Throughout the entire 22-bp oligonucleotide, the electron density for the sugar–phosphate backbone is clearly defined and shows no evidence of significant conformational averaging. Contacts between protein and the DNA backbone are thus apparently unaffected by the orientational averaging. In a parallel study, we have recently determined the co-crystal structure of another b/HLH/Z protein (A.F.-D., P. Pognonec, R. G. Roeder and S.K.B., manuscript in preparation), USF. The asymmetric unit in this co-crystal includes two polypeptide chains and an overhanging 21-bp duplex oligonucleotide, which forms a pseudocontinuous helix and does not show 2-fold averaging. There are no gross differences between this protein–DNA complex structure and the structure we derived from co-crystals of Max interacting with DNA, in which the nucleic acid was partially averaged about a crystallographic symmetry axis. This structural homology also justifies our use of ‘solvent flattening’ in this structure determination and subsequent refinement of only one DNA sequence in the final atomic model.

Structure description

Topological overview. The DNA-binding domain of Max consists of two lengthy α -helices separated by a loop (Figs 2, 3 and 4). The N-terminal α -helix (b/H1) is continuous, and includes residues from the basic and H1 regions. The second α -helix (H2/Z), also continuous, is composed of the H2 and leucine zipper regions. Max binds DNA as a homodimer, and the two monomers fold into a parallel, left-handed, four-helix bundle. Two α -helices, the two basic regions, project from the four-helix bundle towards the DNA and enter the major groove in opposite directions in a manner reminiscent of GCN4^{34,35}. The H1 regions make up half of the four-helix bundle, packing against each other and the two H2 regions. Finally, two Z portions of the second α -helical segment form a parallel, left-handed coiled coil similar in structure to GCN4^{34,35}. Figure 1a illustrates the amino-acid sequences of Max, other b/HLH/Z and some HLH proteins. Detailed analyses of the (Max 22–113)–DNA complex structure presented below confirm predictions that the

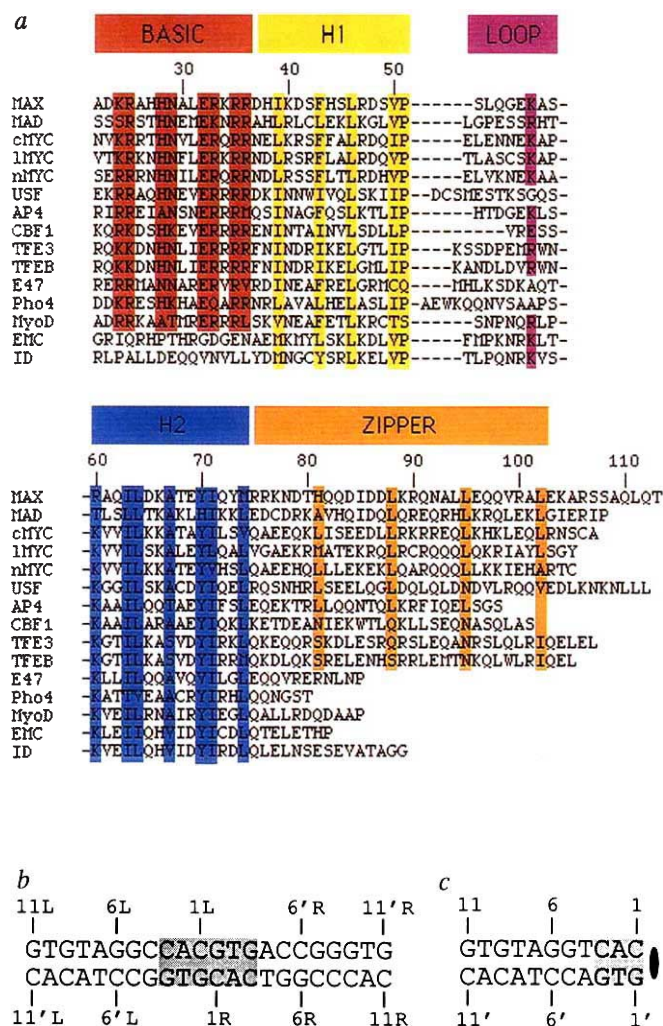


FIG. 1 a, Partial sequence alignment of the DNA binding domain of murine Max protein with b/HLH/Z proteins Mad²³, c-, l- and n-Myc¹⁴, USF⁵⁹, AP4⁶⁰, CBF1⁴⁴, TFE3⁶¹, TFEB⁶², and HLH proteins E47², Pho4⁴⁸, MyoD⁶³, Id⁹, and emc¹⁰. Amino acids in one-letter code are numbered according to the full-size murine Max¹⁴. Conserved residues and the boundaries of each region are highlighted with the same colours as in Figs 2a and 4. Each region of the sequence is named according to convention (b, H1, loop, H2 and Z). The Max amino-acid numbering system is used throughout. b, Sequence of the duplex oligonucleotide used in the crystallization. The core E-box element is shaded. c, Sequence of the DNA model used in the crystallographic analysis, with the position of the molecular dyad indicated by the bold vertical symbol.

TABLE 1 Summary of crystallographic analysis

	Native 1	Native 2	IdU4R	IdU8L	IdU8L, 10L	IdU7'L	IdU10'R
Resolution (Å)	3.3	2.9	3.3	3.3	3.3	3.3	3.3
Reflections (observed/unique)	32,646/3,652	21,448/4,507	34,661/3,592	23,645/3,554	32,012/3,651	30,972/3,683	30,512/3,657
Data coverage (%)	96.1	82.0	94.5	93.5	96.1	96.9	96.2
$R_{\text{sym}}(\%)$ ($ F > 0$)*	8.5	6.5	10.1	11.8	10.9	8.7	11.6
Mean fractional isomorphous difference			18.3	13.0	10.3	9.1	11.3
MIR analysis (15–3.3 Å)							
Phasing power†			0.91	0.48	0.60	0.63	0.53
Mean overall figure of merit			0.58				
Refinement (6–2.9 Å)							
R factor (%)		23.2					
Reflections ($ F > 1\sigma F $)		3,916					
Total number of atoms		1,153					
R.m.s. bond length (Å)‡		0.015					
R.m.s. bond angle (degrees)‡		2.4					
R.m.s. B-factor bonded atoms (Å ²)‡		2.35					

The b/HLH/Z DNA-binding domain of murine Max (Myn) consisting of amino acids Ala 22 to Thr 113 (Max 22–113), derived from a Myn clone¹⁴ with an extra methionine at the N terminus, was overexpressed in *Escherichia coli* (BL21(DE3)pLysS) using the T7 RNA polymerase system⁵³. Cells grown at 30 °C to an absorbance of 1.0 at 595 nm and induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside for 5 h were collected by low-speed centrifugation and lysed by three cycles of freeze-thaw in the presence of 0.1% Nonidet P-40 and DNase I. The soluble fraction was treated with polyethyleneimine⁵⁴, and then fractionated with ammonium sulphate, yielding ~80% pure Max 22–113 protein. Further purification and detergent removal was achieved by cation-exchange chromatography, resulting in protein with an estimated purity of $\geq 98\%$. Laser-desorption mass spectrometry⁵⁵ showed that the purified recombinant protein lacked the N-terminal methionine but was otherwise intact. Further control experiments were done to ensure that the protein used for crystallization was biochemically active. Electrophoretic mobility shift assays¹⁴ verified full DNA-binding activity. Circular dichroism spectroscopy showed that the protein underwent a coil-to-helix folding transition corresponding to an increase in α -helix content of about 18% upon mixing with 1/2 molar equivalents of a 16-bp duplex DNA containing E_{USF} (data not shown). DNA was synthesized using standard phosphoramidite chemistry, and purified by a combination of preparative, denaturing, polyacrylamide gel electrophoresis and anion-exchange chromatography at pH 10.0 and 55 °C. Single-stranded DNA was quantified by ultraviolet spectrophotometry using extinction coefficients that were calculated to incorporate nearest-neighbour contributions⁵⁶, mixed with an equimolar amount of complementary strand, and annealed by heating to 85 °C for 30 min and then cooling to 4 °C at a uniform rate of 0.33 °C min⁻¹. (Max 22–113)-DNA complex, prepared by mixing two molar equivalents of monomer with annealed double-stranded DNA, was concentrated by microfiltration to 0.9 mM. Crystals were grown at 4 °C by microscopic seeding during vapour diffusion equilibration of sitting drops prepared by mixing equal volumes of complex and a reservoir solution consisting of 4.5–7.5% PEG 1000, 5–10% glycerol, 100 mM KCl, 2 mM MgCl₂, and 100 mM sodium cacodylate, pH 5.5–5.75. Hexagonal bars appeared within a few hours of seeding and grew over the course of several days to diameters of typically 0.4 mm and lengths of over 2 mm. Crystals of heavy-atom derivatives were prepared in the same way, using DNA in which 5-iodo-dU had been substituted for T in the positions indicated according to the numbering scheme of Fig. 1b. Care was exercised during the anion-exchange purification step to remove deiodinated DNA using the pK_a difference between bases containing and lacking the halogen. Diffraction data to 3.3 Å resolution were collected using CuK α radiation with a Rigaku RAXIS-IIc imaging plate area detector. Native data to 2.9 Å resolution were collected with 0.91 Å X-radiation at beamline F1 of the Cornell High Energy Synchrotron Source⁵⁷ using imaging plates (between 3.0 and 2.9 Å resolution the data were 61% complete with $\langle I/\sigma(I) \rangle = 2.96$). Each data set was collected from a single crystal maintained at –15 °C. Oscillation photographs were reduced to structure factor amplitudes and scaled using DENZO and SCALEPACK (Z. Otwinowski, personal communication). Additional crystallographic software included the PROTSYS package (G. A. Petsko, personal communication), COMBINE³¹ and NEWHEL92 (R. E. Dickerson, personal communication). The refined iodine atom occupancies and heavy atom derivative phasing powers were lower than is usually found in DNA-protein co-crystal studies, and were inversely correlated with the distance of the halogen site from the crystallographic dyad axis in the middle of the DNA used for crystallization. Presumably, the relatively poor statistics were the result of the twofold averaging of the quasisymmetric DNA in the co-crystal form we investigated. Atomic coordinates and structure factor amplitudes have been submitted to the Brookhaven Protein Data Bank⁵⁸ and will be held for one and four years, respectively. (Atomic coordinates are available immediately to academic users from S.K.B.; non-academic users should contact the Office of Technology Transfer at The Rockefeller University.)

* $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity, and $\langle I \rangle$ is the average intensity obtained from multiple observations of symmetry related reflections. Mean fractional isomorphous difference, $\sum ||F_{\text{PH}}| - |F_{\text{P}}|| / \sum |F_{\text{P}}|$; $|F_{\text{P}}|$, protein structure factor amplitude; $|F_{\text{PH}}|$, heavy atom derivative structure factor amplitude.

† Phasing power, r.m.s. ($|F_{\text{H}}|/E$); $|F_{\text{H}}|$, heavy atom structure factor amplitude, and E , residual lack of closure.

‡ R.m.s. bond lengths and r.m.s. bond angles are the respective root-mean-square deviations from ideal values; r.m.s. thermal parameter is the root-mean-square deviation between the B values of covalently bonded atomic pairs.

b/HLH/Z and HLH proteins constitute a structurally similar group of DNA-binding proteins².

DNA structure. Analysis of the complex reveals only small deviations from canonical B-form DNA³⁶. The average helical twist is 33.5° and the mean rise per base pair is 3.32 Å, implying 10.7 bp per turn. Phasing studies of DNA bending by Max and related proteins suggested bend angles ranging from 50 to 80° (refs 37, 38). In contrast, our structure reveals no net bend. Although this discrepancy might result from crystal packing effects (Fig. 3c), our structure of USF (see above), which has a completely different mode of DNA packing within the co-crystal, also has no net DNA bend.

Monomer structure. The polypeptide backbone adopts α -helix hydrogen-bonding starting with Arg 25, in the basic region, and deviates significantly from it at Ser 49, near the end of H1. The phylogenetically conserved hydrophobic amino acid following this residue (Val 50 in Max) packs against a conserved tyrosine

(Tyr 70) in the H2/Z α -helix. The conserved proline at position 51 provokes a turn in the backbone, starting the eight-amino-acid loop that connects the two α -helical segments. The 43-residue-long H2/Z α -helix begins at the conserved basic residue Arg 60 and extends to Leu 102, the last leucine of the conserved heptad repeat. Of the remaining eleven residues, five have been built as random coil, whereas the C-terminal six amino acids are not visible in the electron density map.

Parallel four-helix bundle. The parallel, left-handed, four-helix bundle, formed by b/H1 and H2/Z derived from each member of the symmetric homodimer, is a new protein fold that creates the hydrophobic core of the Max-DNA complex (Fig. 4b). Without exception, conserved hydrophobic amino acids from H1 and H2 are buried in the interior of the four-helix bundle, where they pack together and stabilize the structure of the homodimer (Figs 1a and 4b). Ile 39, the first of these side-chains, packs against Phe 43, which makes a large number of van der

Waals contacts with H2 side chains, including the methylenes of Arg 60 (Fig. 2c) and Ile 63 and Leu 64' (where prime denotes the other member of the dimer). Leu 46 abuts Ile 63 and Ala 67; Val 50 and Pro 51 pack against Tyr 70. Ile 71 close to the end of H2 packs against its symmetry mate (Ile 71') and Tyr 70'. Met 74 also interacts with its symmetry mate, and is the last

conserved hydrophobic position common to the HLH and b/HLH/Z proteins. Unlike the purely coiled-coil b/Z proteins^{34,35}, homodimers of Max, and by extension homo- and heterodimers of the HLH and other b/HLH/Z proteins, have a globular domain, the four-helix bundle. Stabilization of this novel structure must be due in large part to van der Waals interactions within the conserved, hydrophobic core.

The co-crystal structure presented here also provides some insight into the behaviour of HLH proteins such as Id⁹ and emc¹⁰, which are known as negative regulators and do not bind DNA (Fig. 1a). Neither Id nor emc display any sequence identity or similarity at positions corresponding to those in the basic region α -helix of Max 22–113 that interact with DNA. However, the residues from H1 and H2 that form the hydrophobic core are well conserved; thus, it seems that these proteins can form parallel, four-helix-bundle homo- and heterodimers.

Although our structure of the (Max 22–113)–DNA complex demonstrates a new protein fold, the parallel, four-helix bundle is not unexpected. Experimental observations and model building led some groups to suggest correctly that HLH homodimers form parallel, four-helix bundles^{39–42}. But the (Max 22–113)–DNA complex structure is markedly different from the anti-parallel, four-helix model derived from NMR of a disulphide

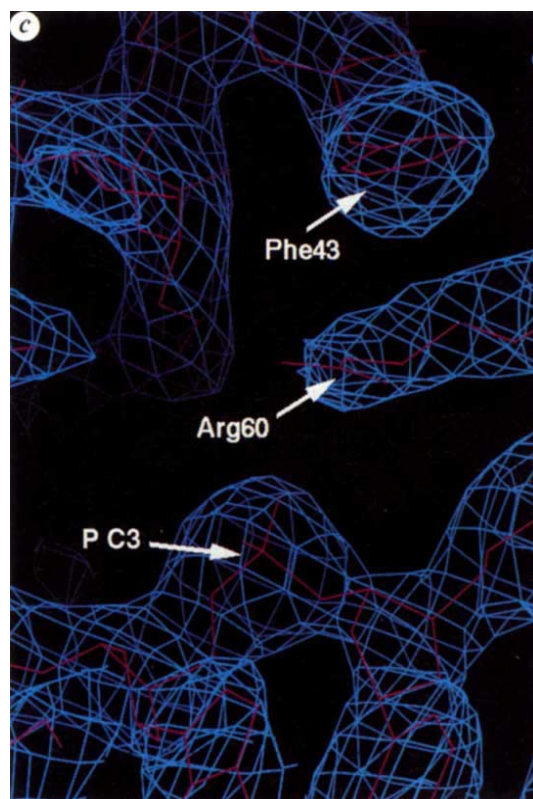
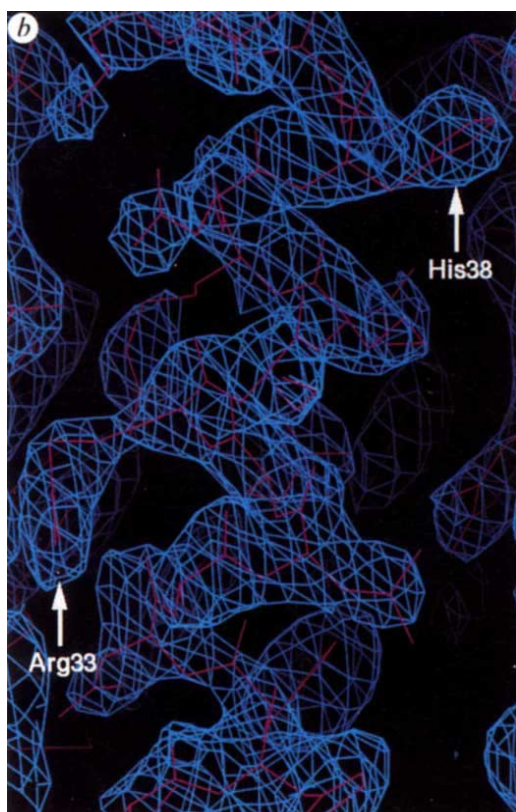
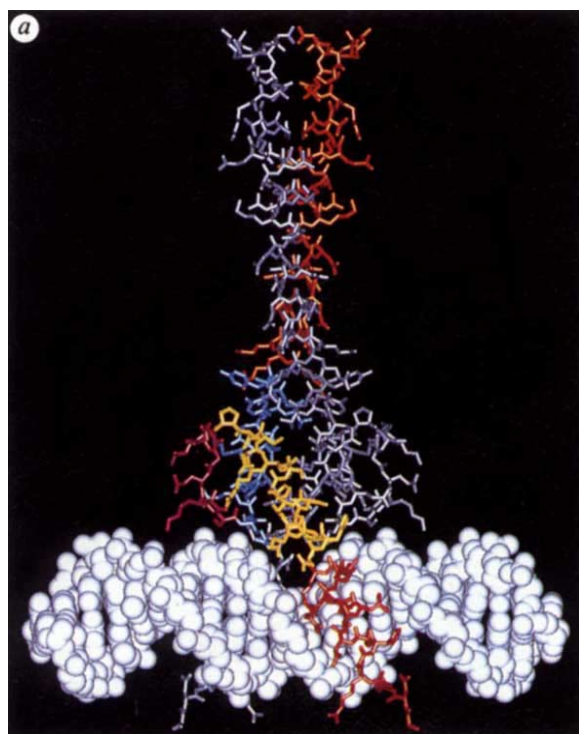


FIG. 2 a, Overview of the (Max 22–113)–DNA complex. Conserved residues are highlighted with the colour scheme used for Fig. 1 in one of the monomers; the symmetry-related molecule is coloured grey for clarity; b plus H1 form a continuous α -helix (red and yellow), as do H2 and Z (blue and orange). The solvent-accessible surface area (H_2O probe size, 1.4 Å) buried upon dimerization is 1,460 Å² per monomer out of 8,080 Å². Binding to DNA results in burial of a further 570 Å² per monomer. b, The final electron density calculated with a $(2|F_{\text{observed}}| - |F_{\text{calculated}}|)$ difference Fourier synthesis is shown in blue, contoured at 1.5 σ , and superimposed with a stick representation of the atomic model in red. This view shows the continuous α -helix consisting of b and H1. c, Electron density calculated as described, showing Phe 43 from H1 packing against Arg 60 at the bottom of H2, which in turn makes a salt bridge with the phosphate of C3.

crosslinked MyoD b/HLH peptide homodimer without DNA⁴³. In our structure, O γ of Ser 49 (Cys 135 in MyoD) is located on the solvent-accessible surface of H1, is 13 Å from its symmetry mate, and could not form a disulphide bond without dramatic alteration in the topologies of both monomers. Mutagenesis of Myc-Max hetero-oligomers⁴² suggests that the parallel, four-helix-bundle structure of the Max homodimer is the fold responsible for DNA binding. These studies demonstrated that all of the conserved, hydrophobic amino acids in H1 and H2 are required for stable Myc/Max-DNA complex formation. Oxidized MyoD is much less active in DNA-binding assays than reduced MyoD⁴³, and we suggest that the NMR structure of the disulphide crosslinked MyoD dimer reflects another possible mode of HLH protein self-association.

Leucine zipper. The leucine zipper of Max is illustrated in Fig. 4c. As predicted from sequence similarity, the structure is a

left-handed coiled coil of two right-handed α -helices. In addition to the classical Leu-Leu and Ile-Ile interactions, it shows an interesting His-His packing at position 81, and a remarkable Gln-Asn-Gln-Asn tetrad at positions 91 and 92. A number of salt-bridge and hydrogen-bond interactions occur within and between the α -helices forming the coiled coil. The N-terminal two thirds of the coiled coil closely resembles the structure of GCN4^{34,35}. Beyond Leu 102, the last conserved member of the heptad repeat, the coiled coil ends abruptly as the polypeptide chain becomes random coil. Although b/HLH/Z dimers may interact with other proteins through the leucine zipper, there is no clear pattern of conserved residues displayed on the exposed surface of the coiled coil.

Loop. HLH and b/HLH/Z proteins vary in the sequence, amino acid composition, and length of the loop connecting H1 and H2. Homologous protein sequences include loops ranging from

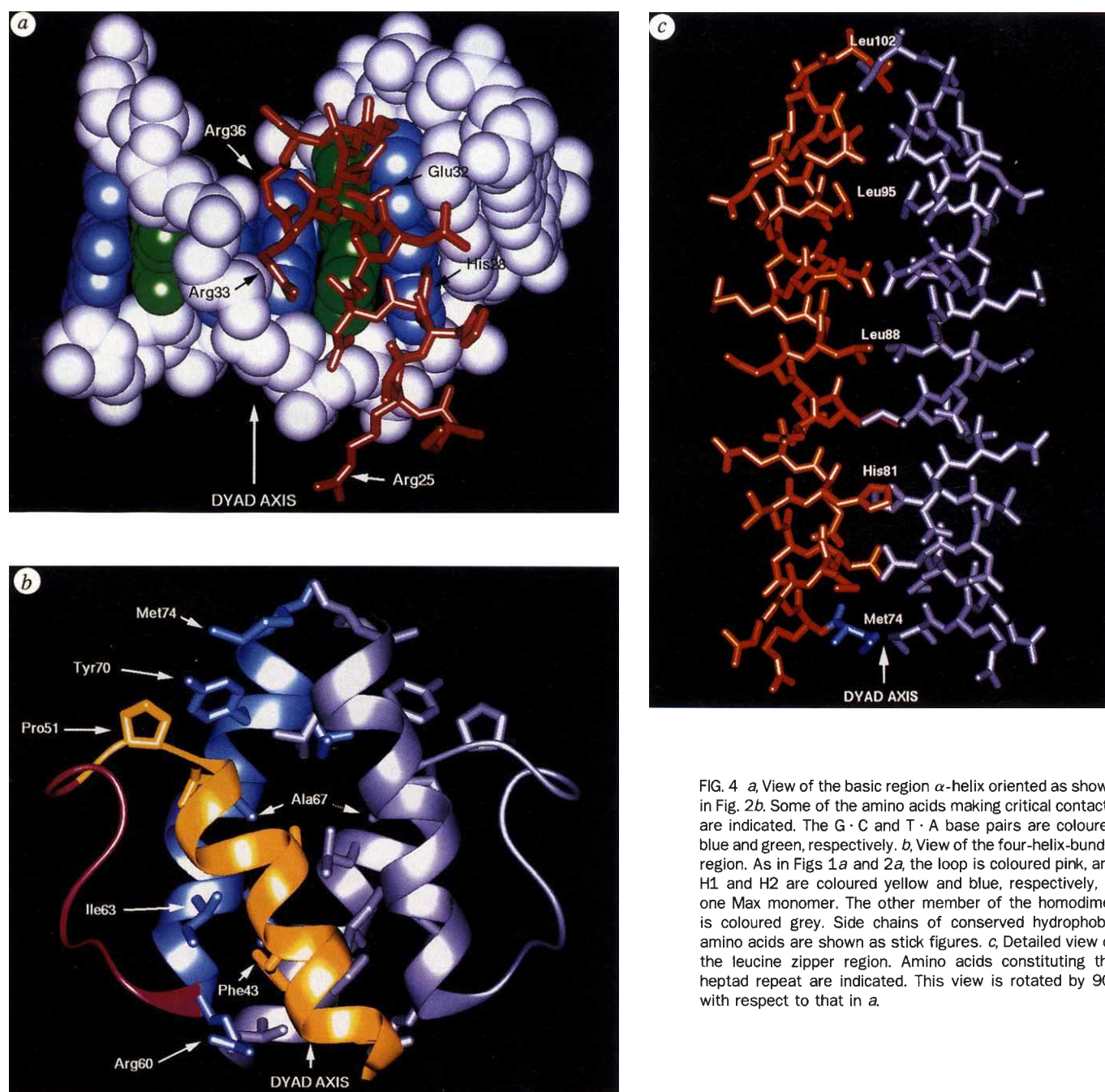


FIG. 4 *a*, View of the basic region α -helix oriented as shown in Fig. 2*b*. Some of the amino acids making critical contacts are indicated. The G $\cdot$ C and T $\cdot$ A base pairs are coloured blue and green, respectively. *b*, View of the four-helix-bundle region. As in Figs 1*a* and 2*a*, the loop is coloured pink, and H1 and H2 are coloured yellow and blue, respectively, in one Max monomer. The other member of the homodimer is coloured grey. Side chains of conserved hydrophobic amino acids are shown as stick figures. *c*, Detailed view of the leucine zipper region. Amino acids constituting the heptad repeat are indicated. This view is rotated by 90° with respect to that in *a*.

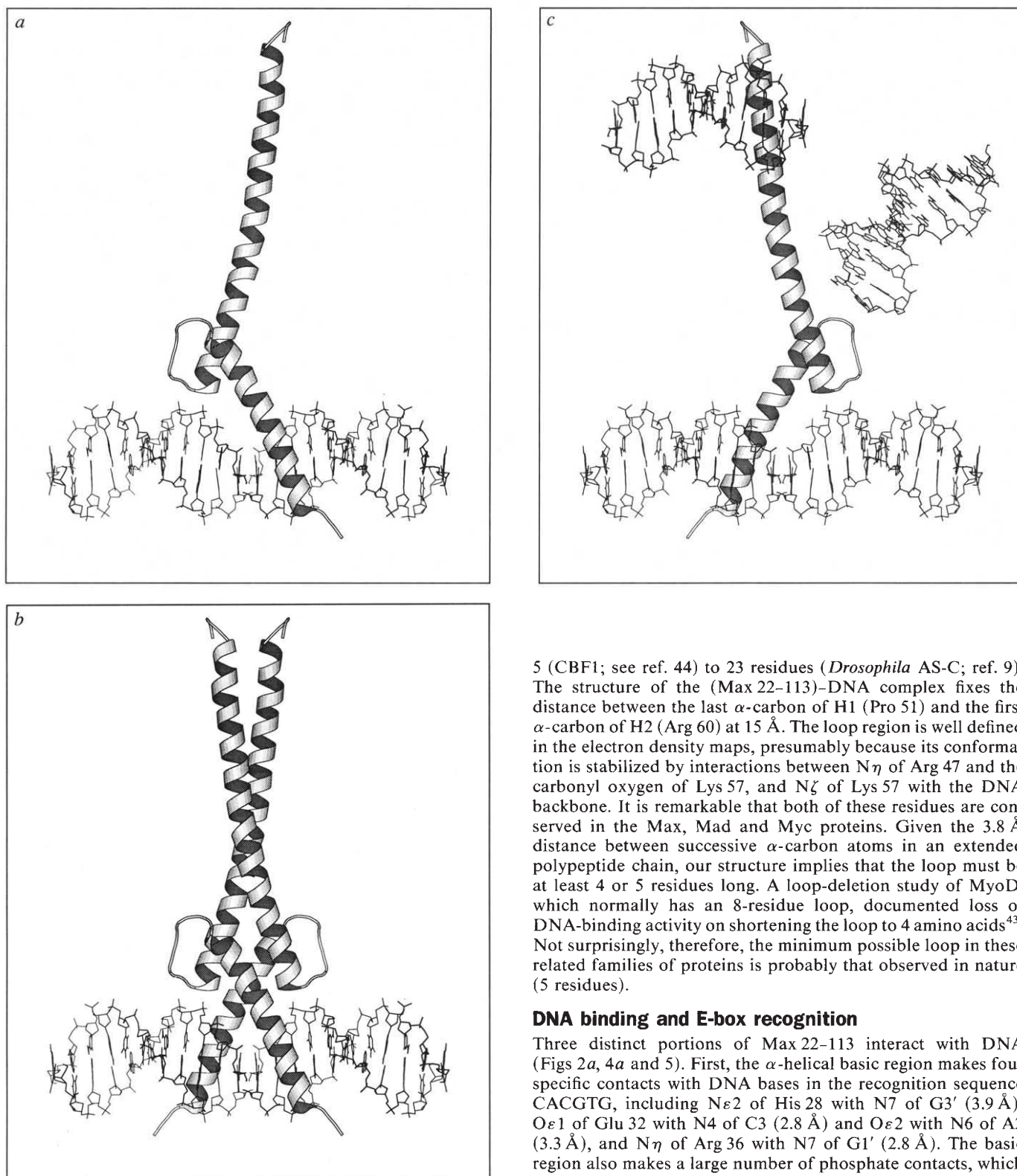


FIG. 3 Richardson-type⁶⁴ cartoon representations of the (Max 22-113)-DNA complex. Figures were generated with the program MOLSCRIPT⁶⁵. *a*, Schematic view of a monomer interacting with 22 bp of DNA. *b*, Same view as in *a*, showing the homodimer-DNA interaction. *c*, Max 22-113 monomer with DNA, showing crystal packing contacts made by two additional oligonucleotides from neighbouring asymmetric units. Although the 22-bp oligonucleotide was designed for crystallization using the so-called 'rule of 7'⁶⁶, the DNA does not form a pseudocontinuous helix within the crystal. Instead, the base pair at the end of one DNA duplex packs against the loop region, while another DNA makes nonspecific contacts with the leucine zipper. This view corresponds to that in *a*, rotated by 180° about the vertical.

5 (CBF1; see ref. 44) to 23 residues (*Drosophila* AS-C; ref. 9). The structure of the (Max 22-113)-DNA complex fixes the distance between the last α -carbon of H1 (Pro 51) and the first α -carbon of H2 (Arg 60) at 15 Å. The loop region is well defined in the electron density maps, presumably because its conformation is stabilized by interactions between N η of Arg 47 and the carbonyl oxygen of Lys 57, and N ζ of Lys 57 with the DNA backbone. It is remarkable that both of these residues are conserved in the Max, Mad and Myc proteins. Given the 3.8 Å distance between successive α -carbon atoms in an extended polypeptide chain, our structure implies that the loop must be at least 4 or 5 residues long. A loop-deletion study of MyoD, which normally has an 8-residue loop, documented loss of DNA-binding activity on shortening the loop to 4 amino acids⁴³. Not surprisingly, therefore, the minimum possible loop in these related families of proteins is probably that observed in nature (5 residues).

DNA binding and E-box recognition

Three distinct portions of Max 22-113 interact with DNA (Figs 2*a*, 4*a* and 5). First, the α -helical basic region makes four specific contacts with DNA bases in the recognition sequence CACGTG, including N ϵ 2 of His 28 with N7 of G3' (3.9 Å), O ϵ 1 of Glu 32 with N4 of C3 (2.8 Å) and O ϵ 2 with N6 of A2 (3.3 Å), and N η of Arg 36 with N7 of G1' (2.8 Å). The basic region also makes a large number of phosphate contacts, which span the entire backbone of the recognition element. In the (Max 22-113)-DNA co-crystals studied here, N ζ of Lys 24 interacts with the phosphate backbone of a neighbouring DNA; however, in the absence of this crystal contact it could make a salt bridge with the phosphate group of A7. N-terminal extensions of the protein, not included in this study, may also make DNA contacts. Second, Lys 57 in the loop region of Max stabilizes the meandering path of the loop, which extends across the adjacent minor groove. This structural feature allows the lysine side chain to straddle the minor groove, making a salt bridge with the phosphodiester backbone on its opposite side

(Fig. 2a). In the case of very long loops, contacts with the adjacent minor (or even major) grooves are possible and could represent a means of generating DNA-binding selectivity outside the central CACGTG element. Indeed, some workers^{8,25,45} have suggested that different HLH and b/HLH/Z proteins have preferences for flanking bases. Third, Arg 60, which is located at the start of H2, makes a side-chain contact with the phosphate of C3 (Fig. 2c) and a main-chain amide contact with the phosphate of T4. Only these three types of protein-nucleic acid contact are seen in our structure of the USF-DNA complex, making it unlikely that the partial, orientational averaging of the DNA in the Max co-crystals obscured anything of biological importance.

The minimal DNA element required for specific binding by the HLH and b/HLH/Z transcription factors is the so-called 'E-box' CANNTG^{25,45-49}. Site-directed mutagenesis of members of both protein families demonstrated that conserved amino acids in the basic region are involved in DNA binding^{6-8,25}. Sequence comparisons of family members showed that the presence of an arginine at position 36 in Max distinguishes proteins that bind to CACGTG (class B) from those which have a hydrophobic or polar amino acid at position 36 and recognize CAGCTG (class A)⁵⁰. Studies of the yeast HLH protein Pho4 documented that the conserved glutamate (residue 32 in Max) is essential for binding; substitution for glutamine, aspartate or leucine, abolished DNA binding⁸.

The (Max22-113)-DNA complex structure clarifies the mechanisms of E-box recognition by the α -helical basic region.

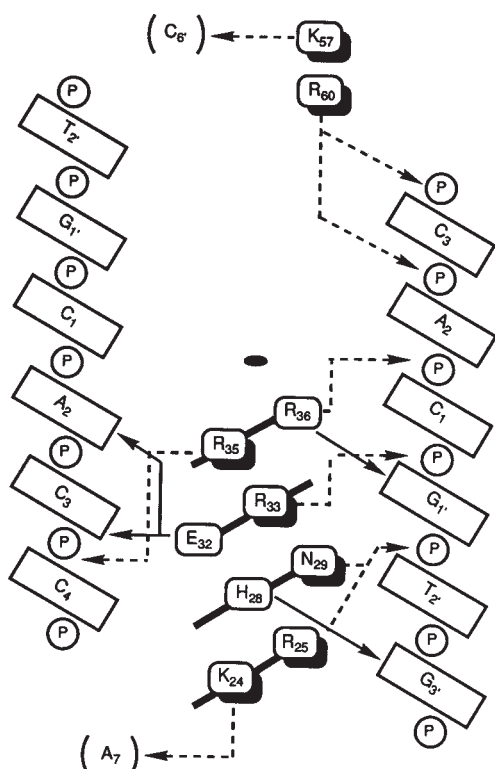


FIG. 5 Schematic summary of DNA contacts made by Max 22-113. For clarity, only contacts made by one of the two dyad-related protein monomers are illustrated. Contacts of amino-acid side-chains to nucleotide bases are represented by continuous arrows; contacts with the phosphate backbone by broken arrows; amino acids making phosphate contacts are shaded. Contacts outside the boundaries of the figure are indicated in parentheses. The position of the dyad axis is indicated by a black ellipse symbol. This view is drawn from the vantage point of the DNA major groove looking out towards the surface of the basic region α -helix, and is the opposite of the views depicted in Figs 2a, 3a and b, and 4a.

All the conserved amino acids in the basic region make either base or phosphate contacts (Figs 2c, 4a and 5). Moreover, the structural basis of class A versus B discrimination is revealed by our study. Arg 36 interacts with the base of the central G (G1') and with the phosphate between C1 and A2 (Figs 1c and 5), across the crystallographic dyad. Glu 32 makes a bidentate contact with the bases of A2 and C3 within the E-box. Mutation of residue 32 to glutamine abolishes DNA binding⁸. Its location in the major groove is incompatible with DNA recognition by the shorter acidic side chain of aspartate. Mutagenesis of the conserved basic residue (Arg or Lys) at the beginning of H2 in E47 to alanine abolishes both homodimerization and DNA binding⁵. In the (Max 22-113)-DNA complex, this basic residue (Arg 60) tethers H2 to DNA by means of two phosphodiester backbone contacts and stabilizes the interaction of H1 with H2 by packing against the conserved hydrophobic residue in H1 (Phe 43) (Fig. 2c). Elimination of the positive charge and the hydrophobic methylene groups by mutation from a basic residue to alanine would interfere both with DNA binding and dimerization.

In conclusion, the co-crystal structure of Max 22-113 presented here provides a starting point for further crystallographic, biochemical and genetic studies of the biologically important b/HLH/Z and HLH families of structurally related transcription factors. In particular, it should now be possible systematically to determine the structural bases and mechanisms by which Max plays a central part in mediating the biological effects of the Myc/Max and Mad/Max heterodimers, how other b/HLH/Z and HLH proteins discriminate among each other in oligomerizing, and how HLH proteins interact with b/Z proteins^{51,52}. □

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Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation

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The proteins Grb2–Sem-5, Shc and Sos have been implicated in the signalling pathway from tyrosine kinase receptors to Ras. Grb2–Sem-5 binds directly to murine Sos1, a Ras exchange factor, through two SH3 domains. Sos is also associated with ligand-activated tyrosine kinase receptors which bind Grb2–Sem-5, and with the Grb2–Sem-5 binding protein, Shc. Ectopic expression of *Drosophila* Sos stimulates morphological transformation of rodent fibroblasts. These data define a pathway by which tyrosine kinases act through Ras to control cell growth and differentiation.

MANY oncogenes encode protein tyrosine kinases (PTKs) which transmit signals regulating cellular growth and differentiation from the cell surface^{1,2}. Another class of oncoprotein implicated in the process is the Ras family of low- M_r GTPases^{3–7}. Inhibition of Ras function by microinjection of anti-Ras antibodies blocks the growth and transformed phenotype of tyrosine-kinase-transformed fibroblasts, indicating that Ras functions downstream from activated tyrosine kinases⁸, and many tyrosine kinase receptors when activated, also activate Ras⁹.

Genetic analysis of eye development in *Drosophila melanogaster* has shown that the Sevenless tyrosine kinase receptor is critical for induction of R7 photoreceptor neurons¹⁰, making it possible to define other elements in the signalling pathway triggered by sevenless¹¹. The *Son of Sevenless* gene (*Sos*) has thus been shown to function downstream from *Sevenless* and downstream from a second tyrosine kinase receptor, the *Drosophila* epidermal growth factor receptor homologue (DER)^{12,13}. *Sos* is homologous to exchange proteins that activate Ras by inducing loading of GTP^{13,14}. A second downstream gene encodes the *Drosophila* Ras protein DRas1 (ref. 13) providing strong evidence that in insect cells, sevenless and DER use the Ras pathway to transduce growth and differentiation signals.

The situation is similar in the system governing vulval development in *Caenorhabditis elegans*^{15,16}, where the locus *let-23* encodes the *C. elegans* homologue of the EGF receptor¹⁷, and a second gene (*let-60*) specifying a downstream function encodes a Ras protein^{18,19}. Yet another gene in this pathway,

termed *sem-5*, encodes a small protein consisting entirely of two src-homology-3 (SH3) domains flanking a src-homology-2 (SH2)²⁰ domain. Mutations in *sem-5* can be rescued by activated *ras* alleles indicating that this protein functions upstream of the *let-60* Ras protein (ref. 20 and S. G. Clark and H. R. Horvitz, personal communication).

Sem-5 is thought to function as an adapter that assembles other proteins into multi-protein complexes^{20,21} by means of its SH3 and SH2 domains, which bind short proline-rich sequence motifs and phosphotyrosine-containing peptides, respectively. SH2 domains, such as that in *Sem-5*, are found in certain effector proteins and have been implicated in the recruitment of these effectors into complexes with autophosphorylated tyrosine kinase receptors²².

In mammals, Ras guanine nucleotide exchange activity is stimulated by tyrosine kinase receptors, including the *trk*-encoded nerve growth factor receptor, the EGF receptor (EGF-R), and the insulin receptor^{23–25}. A mammalian homologue of *Sem-5*, designated Grb2, has been cloned in screens both for proteins which bind to the autophosphorylated EGF-R cytoplasmic tail²⁶ and for SH2-containing proteins²⁷; mammalian homologues of the *Drosophila* *Sos* gene (termed mSos1 and mSos2) are also known²⁸. The interactions between mammalian tyrosine kinases, Grb2, the mammalian *Sos* proteins and Ras can now be analysed, together with their involvement in signal transduction. Here we describe physical complexes between several of these signalling proteins strongly supporting the hypothesis that tyrosine kinase receptors introduce mitogen signals into the Ras signalling pathway by forming multiprotein complexes with the Grb2 and mSos proteins. One implication of these interactions has been confirmed through study of the

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effects of ectopically expressed *Drosophila* Sos protein in rodent fibroblasts.

Association of mSos with Grb2

Genetic analyses suggest that the *Drosophila* Sos and *C. elegans* Sem-5 proteins function near one another in their respective signalling cascades, downstream from receptor tyrosine kinases but upstream from Ras. We therefore investigated whether Grb2 and mSos formed physical complexes *in vivo* by tagging the Grb2 protein at its C terminus with the 9E10 Myc epitope to give the Grb2myc chimera²⁹. This gene was subcloned into the pBabepuro retroviral vector and packaged to produce retroviral stocks³⁰. Rat1 cells were then infected with this virus, with control virus encoding untagged Grb2 or with control pBabepuro virus and puromycin-resistant populations from each infection were selected. Cell clones infected with the Grb2myc virus were also isolated. Western blot analysis of cell lysates using the 9E10 antibody revealed the expected 26K Grb2myc protein in cells infected with Grb2myc (Fig. 1a). The

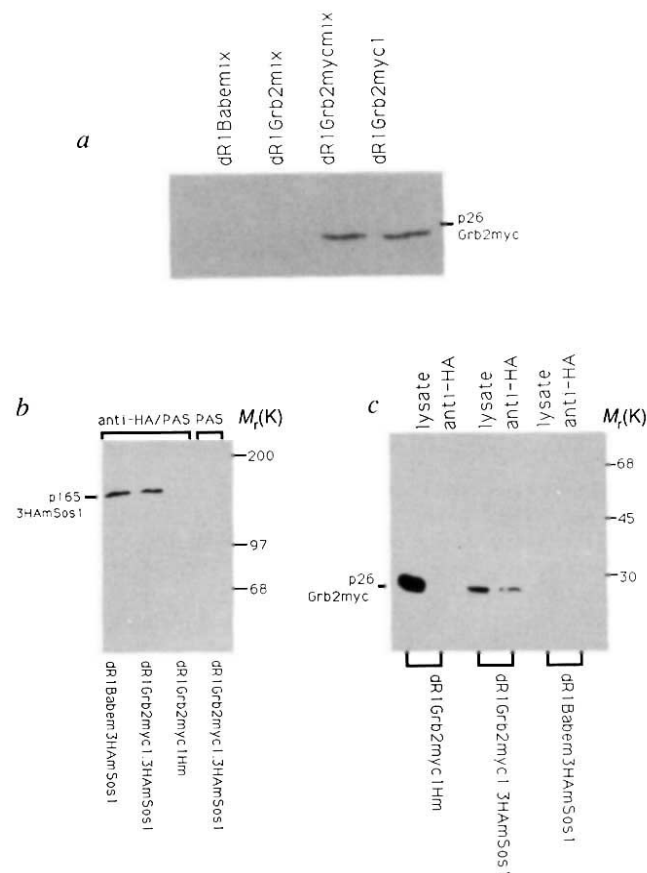
cloned cell line dR1Grb2myc1, which expresses tagged Grb2, and the control-infected cell population (dR1Babemix) were used for further analysis.

The mouse Sos protein (mSos1) was provided with a distinct antigenic tag by fusing the mSos1 reading frame at its N terminus to three copies of the influenza virus haemagglutinin (HA) epitope³¹. This peptide is efficiently recognized by monoclonal antibody 12CA5. The resulting chimaeric gene (3HAMsOs1) was introduced into the pBabehygro retrovirus transducing vector, which carries a hygromycin resistance marker instead of the puromycin marker present in the Grb2 vector³⁰. Retroviral stocks were generated from the pBabehygro3HAMsOs1 and control pBabehygro vectors, which were used to infect dR1Grb2myc1 or dR1Babemix cells, and infected populations were selected in the presence of hygromycin.

Lysates of the resulting double drug-resistant cell populations were then immunoprecipitated with the anti-HA antibody 12CA5, and the precipitates analysed by western blot analysis using anti-HA (Fig. 1b). The 165K HA-tagged 3HAMsOs1 pro-

FIG. 1 *In vivo* association of Grb2 and mSos1. **a**, Western blot analysis of Rat 1 cells infected with Grb2myc virus. **b**, Western blot of anti-HA immunoprecipitates probed with 12CA5 anti-HA antibody. **c**, Western blot of whole-cell lysates and anti-HA immunoprecipitates probed with 9E10 anti-myc antibody.

METHODS. **a**, The human Grb2 gene was cloned from HT1080 complementary DNA by PCR using the 5' primer GCTAAAGGATCCCTCAGAATGGAAGCCATCGCC (where the natural ATG start codon is underlined) and the 3' primer GGATTCTCTAGAGTCGACTCAGACGTTCGGTTCACGGGGT. The PCR product was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI-*Xba*I-cut pBSK+. After sequencing clones and subcloning of non-mutant fragments, the vector pBSKGrb2A1 was generated which encoded full length Grb2. The *Bam*HI-*Sal*I Grb2 fragment was then subcloned into *Bam*HI-*Sal*I-digested pBabepuro to generate the retroviral vector pBabeGrb2A. The myc-tagged Grb2 gene was generated in the same way except PCR used the 3' primer GGATTCTCTAGAGTCGACTCACAAGTCTTCTTCAGAAATAAGCTTTTGTTCGACGTTCGGTTCACGGGGT, where the underlined segment encodes the myc epitope. The vector pBSKGrb2mycAAA was generated which encoded full-length Grb2myc. The authenticity of this gene was also confirmed by sequence analysis. The *Bam*HI-*Sal*I Grb2myc fragment from pBSKGrb2mycAAA was also subcloned into *Bam*HI-*Sal*I-digested pBabepuro to generate pBabeGrb2mycA. pBabepuro, pBabeGrb2A and pBabeGrb2mycA were transiently transfected into the retroviral packaging line GP+E (ref. 40) and viral supernatants collected 48 h later. Rat1 cells were infected with these viral stocks and selected in 5 μ g ml⁻¹ puromycin. The cell cultures dR1Babemix, dR1Grb2mix and dR1Grb2mycmix are polyclonal mixtures of puro-resistant cells. dR1Grb2myc1 was isolated as a cloned line. Confluent monolayers of these lines were collected in 1 ml of lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 10 μ g ml⁻¹ aprotinin, 1 mM PMSF, 1 mM Na₃VO₄) per 9-cm dish and clarified by centrifugation. Each lysate (20 μ l) was boiled in SDS-PAGE sample buffer and resolved on a 12% SDS-PAGE gel. The gel was electroblotted onto nitrocellulose. This filter was then blocked in 5% dry milk powder, 0.05% Tween 20, PBS, washed in 1% dry milk powder, 0.05% Tween 20, PBS, probed with 1 μ g ml⁻¹ of 9E10 monoclonal antibody in wash buffer, washed, incubated in 1/5,000 dilution of Pierce Goat anti-mouse Fc horseradish peroxidase (HRP)-conjugated antiserum, washed and developed by ECL (Amersham). **b** and **c**, The triple HA-tagged mSos1 gene was constructed from its fragments. The 5' end of the gene was replaced by the sequence AAGCTTAGATCTACCATGGGCGGCGCTTGCTCCACCTCATC by PCR-mediated mutagenesis, where the underlined ATG is the start codon. This altered 5' end contained a *Not*I restriction site into which the triple HA epitope was cloned³¹. A retroviral expression vector (pBabeH3HAMsOs1A) was generated by cloning fragments of the gene from the *Bgl*II site present in the 5' PCR primer to a *Sal*I site downstream of the gene (originally in the pBSIlm-Sos1.385 partial cDNA-containing plasmid²⁸) into pBabehygro digested with *Bam*HI and *Sal*I. pBabeH3HAMsOs1A or pBabehygro were transiently transfected into GP+E packaging cells and viral supernatants collected. dR1Babemix cells were infected with the H3HAMsOs1 viral stock and the polyclonal population dR1Babem3HAMsOs1 selected in 100 μ g ml⁻¹ hygromycin. dR1Grb2myc1 cells were infected with either H3HAMsOs1 viral stocks or hygro control viral stocks. These cells were then selected in 100 μ g ml⁻¹ hygromycin and the polyclonal populations dR1Grb2myc1.3HAMsOs1 and dR1Grb2myc1Hm isolated separately. Four



confluent 9-cm dishes of each line were serum starved overnight in DME, 0.2% FCS, treated with 100 μ M Na₃VO₄ for 30 min followed by 100 ng ml⁻¹ EGF for 5 min. The cells were then washed with ice-cold PBS and lysed in 1 ml of 30 mM HEPES pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mg ml⁻¹ BSA, 1 mM Na₃VO₄, 2 mM para-nitrophenylphosphate, 10 mM NaF, 25 μ M phenyl arsine oxide, 25 μ g ml⁻¹ leupeptin, 25 μ g ml⁻¹ trypsin inhibitor, 25 μ g ml⁻¹ pepstatin A, 25 μ g ml⁻¹ aprotinin, 10 mM benzamide, 1 mM PMSF. Lysates were clarified and incubated with 2 μ g of anti-HA antibody covalently coupled to protein A-Sepharose⁴¹ for 2 h at 4 °C. Immunoprecipitates were washed four times in 1 ml of PBS, 0.1% Triton X-100, 0.005% SDS, 0.25 M NaCl, 1 mM Na₃VO₄, 1 mM PNPP. Samples were run on 7 or 12% gels and immunoblotted with anti-HA (1 μ g ml⁻¹) or 9E10 anti-myc (1 μ g ml⁻¹), followed by anti-mouse IgG Fc-HRP (Pierce) with visualization by ECL (Amersham). The anti-HA precipitate from cell line dR1Grb2myc1.3HAMsOs1 in c was from 450 μ l of extract, whereas 30 μ l of extract was loaded in the total lysate track.

tein was easily observed in cells infected with the 3HAmSosl virus but not in cells infected with control virus. As expected, the 26K tagged Grb2 protein was also present in hygromycin-resistant derivatives of the dR1Grb2myc1 cell line (Fig. 1c). When anti-HA immunoprecipitates from these lysates were western blotted with the anti-myc antibody, however, the Grb2myc protein was only precipitated from cells that expressed the 3HAmSosl protein, indicating that Grb2 and mSosl do associate *in vivo* (Fig. 1c).

SH3 binding

To understand the interaction between Grb2 and mSosl in more detail, we made glutathione-*S*-transferase (GST) fusion proteins with Grb2myc, the mSosl N-terminal domain, the mSosl central domain (homologous to the yeast CDC25 Ras exchanger), a longer version of the mSosl central domain, and the mSosl C-terminal domain. Expression of the mSosl fusions or a non-chimaeric GST control protein was then induced in bacteria

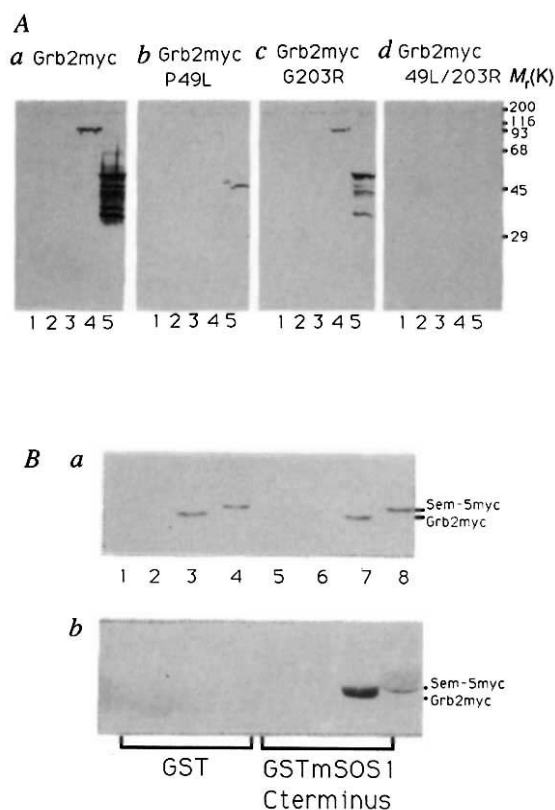
and the lysates analysed by far-western blotting using a purified GSTGrb2myc fusion protein. The Grb2 protein was detected using the 9E10 anti-myc antibody.

Grb2 bound specifically to the C-terminal domain of mSosl (amino-acids 1,135–1,336), indicating that no additional partners are required to couple these two proteins (Fig. 2A, a). This binding was specific to Grb2, because a nonfunctional myc-tagged Grb2 mutant described below did not bind to mSosl fragments (Fig. 2A, d). Grb2 also bound to an overlapping fusion protein containing residues 613–1,166 of mSosl, but not to a smaller protein containing residues 613–1,135, indicating that a binding site for Grb2 is located between residues 1,135 and 1,166 of mSosl. As these 31 amino acids contain a proline-rich stretch which is similar in sequence to recently described SH3-binding motifs³², we speculated that Grb2 uses one or both of its SH3 domains to bind to this proline-rich sequence in mSosl.

Nonfunctional alleles of *sem-5* have been found to contain

FIG. 2 A, Binding of GSTGrb2myc fusion proteins to mSosl fusion proteins immobilized on nitrocellulose filters. Replicate filters with bacterial lysates containing: lanes 1, GST protein; 2, GSTmSoslNterminus; 3, GSTmSoslcentral; 4, GSTmSoslcentral+31; 5, GSTmSoslCterminus. Filters were probed with a, GSTGrb2myc; b, GSTGrb2myc49L; c, GSTGrb2myc203R; d, GSTGrb2myc49L/203R. B, Analysis of solution binding between Grb2/Sem-5-myc proteins and GSTmSoslCterminus beads. a, Western blot of total cell lysates for myc-tagged proteins produced in Cos1 cells transfected with: lanes 1 and 5, pSVE+; lanes 2 and 6, pSVEGrb2B; lanes 3 and 7, pSVEGrb2mycA.6A; lanes 4 and 8, pSVEsem-5mycB. b, Western blot of protein samples 1 to 8 from a precipitated with either GST beads (lane 1–4) or GSTmSoslCterminus beads (lanes 5–8).

METHODS. A, Fragments of mSosl were cloned into derivative pGEX vectors as follows. GSTmSoslNterminus: the N terminus of mSosl was constructed from three partial clones²⁸ and the starting sequence replaced by PCR-mediated mutagenesis using the 5' oligonucleotide ACCGCCAAGCTTAGA-TCTTAGCCTAAGGCAATGCTT, where the underlined ATG is the natural start codon. PCR-derived fragments were sequenced. The N-terminal sequences beginning at the *Bgl*II site in the above primer and ending at the unique *Bam*HI site was cloned into *Bam*HI-digested pGEX20T. This fusion protein encodes the first 615 amino acids of mSosl. GSTmSoslcentral: this vector was made by subcloning the *Bam*HI-*Bgl*II (nucleotide (nt) 3,437) fragment of mSosl into pGEX30X. It encodes amino acids 615–1,135. GSTmSoslcentral+31: the central region of mSosl from the unique *Bam*HI site to the *Pvu*II site at nt 3,534 representing amino acids 613–1,166 was cloned into modified pGEX30X, starting at the *Bam*HI site. GSTmSoslCterminus: the C terminus of mSosl from amino acid 1,135 to 1,336 was subcloned on a *Bgl*II-*Xho*I fragment into *Bam*HI-*Xho*I-digested pGEX20T. The GSTGrb2myc-encoding vector was made by subcloning the *Bam*HI-*Xba*I fragment containing Grb2myc from pSVEGrb2mycA.6A into *Bam*HI-*Xba*I-digested pGEX20T. The resulting plasmid codes for full-length Grb2myc fused to GST. The pSVEGrb2mycA.6A plasmid was itself made by inserting the *Bam*HI-*Sac*I fragment from pBSKGrb2mycAAA (described in Fig. 1) into *Bam*HI-*Sac*I-cut pSVE+ (ref. 42). All mutants were made by phagemid rescue of pSVEGrb2mycA.6A using the BioRad mutagenesis kit, following manufacturer's instructions. Mutants were sequenced and were subcloned into pGEX20T. Induced bacterial cultures expressing GSTmSosl fusion proteins or pGEX30X-encoded GST were centrifuged, resuspended in protein sample buffer, boiled, separated on 12% SDS-PAGE gels and transferred to nitrocellulose using standard techniques⁴³. Filters were blocked with 5% dry milk powder, 0.05% Tween 20 in PBS for 1 h and then incubated with 1 μ g ml⁻¹ purified Grb2myc fusion proteins in 1% dry milk powder, 0.05% Tween 20, PBS; the myc epitope was detected with 9E10 anti-myc antibody as in Fig. 1a. A western blot of these samples using anti-GST antiserum confirmed that fusion proteins of the expected sizes were present in all tracks (data not shown). The full-length C-terminal fusion protein is insoluble in bacteria and despite direct lysis of induced cultures in SDS-PAGE sample buffer with immediate boiling, degradation of this protein was reproducibly observed. Grb2myc fusion proteins were purified by binding to glutathione agarose and eluted with glutathione⁴³. B, Cos1 cells were transfected with 20 μ g of plasmid DNA encoding tagged Grb2-sem-5 genes or control vectors by calcium phosphate coprecipitation⁴⁴. About 48 h later, cells were washed in cold PBS and collected in lysis buffer (Fig. 1a) and centrifuged. Clarified lysates were either directly run on 12% SDS-PAGE gels, transferred to nitrocellulose and western blotted for the myc-tagged protein (a) as described in Fig. 1 (1/50th of lysate) or precipitated with beads (GST or



GSTmSoslCterminus) for 1 h (b). The precipitated proteins were then washed (4 $\times$ 1 ml 20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100), separated on a 12% SDS-PAGE gel, transferred and western blotted for the myc epitope (a) (49/50ths of lysate). The pSVEGrb2mycA.6A plasmid used to direct synthesis of myc-tagged Grb2 was made as described in methods from A. The pSVEGrb2B plasmid was made by subcloning the *Bam*HI-*Sac*I fragment from pBSKGrb2A1 (described in Fig. 1) containing full length Grb2 into *Bam*HI-*Sac*I-digested pSVE+. The myc-tagged *sem-5* gene was synthesized by PCR with the 5' primer GCTAAAGGATCCGAATCCG-AAGTTTGAGG and the 3' primer GGATTCTCTAGAGTCGACTACAAGTCT-TCTTCAGAAATAAGCTTTTGTTCATTCCAAAGTTGAAGCC (where the underlined sequence is the antisense sequence coding for a C-terminal 9E10 myc epitope) using pBSK-sem-5 as a template. The PCR product was digested with *Bam*HI and *Xba*I and cloned into *Bam*HI-*Xba*I-digested pBSK+ to yield pBSKsem-5mycA. The majority of the *sem-5* coding sequence was then replaced by subcloning the *Eco*RI to *Sna*BI fragment of wildtype *sem-5* from pBSK-sem-5 into *Eco*RI (partially digested) to *Sna*BI-digested pBSKsem-5mycA which generated pBSKsem-5mycA1. The *Sna*BI to stop codon including the myc epitope tag region were checked by sequencing. Finally, the tagged gene was cut out from pBSKsem-5mycA1 on a *Bam*HI-*Sac*I fragment and ligated into *Bam*HI-*Sac*I-digested pSVE+ plasmid.

TABLE 1 Transforming potential of DSos

Hygro	
Hygro 3 viral stock	< 0.00035 foci per hygro-resistant colony
Neu	
Neu4 viral stock	0.39 foci per hygro-resistant colony
SOS	
H.Sos.B viral stock	0.034 foci per hygro-resistant colony
H.Sos.J viral stock	0.025 foci per hygro-resistant colony

Rat 1 cells were infected with retroviral supernatants from cloned producer cell lines shedding either the pBabehygro virus or pBabehygro derivatives that encode the activated *neu* gene or DSos. After infection, cells were either grown to confluence to score for the production of transformed foci or cultured in the presence of $100 \mu\text{g ml}^{-1}$ hygromycinB to determine infection frequency. Hygromycin-selected plates were stained on day 14 and drug-resistant colonies counted. Focus-forming assays were stained on day 21 after infection. Transformed foci were counted from the stained plates. Foci resulting from infection by the *neu* virus were typically larger than DSos-induced foci and were apparent earlier. Producer lines were obtained by transfection of pBabe-vectors into the Ω E packaging cell line³⁰, followed by selection of hygromycin-resistant transfectant clones which were screened for production of hygromycin resistance-encoding viral supernatants. Highest titre cloned producer lines were used as a source of supernatants for transformation assays. The pBabeHNeuNT retroviral vector was made by subcloning the blunted *HindIII*-*SalI* Neu-fragment originally from pSV2NeuNT (ref. 46) into *SnaBI*-digested pBabehygro³⁰. The pBabeHSosA vector is also a derivative of pBabehygro. The natural 5' end of DSos was replaced by the sequence CATTCGGATCCAGAACCATGTTCTC-GGGGCCAGCGGCCAT through polymerase chain reaction (PCR)-mediated mutagenesis. The PCR-derived 5' fragment was digested with *Bam*HI and *Pst*II. This small fragment containing the first 53 codons was cloned together with the *Pst*II (partially digested) to *Dra*I fragment containing the remainder of the gene into *Bam*HI-*Sna*BI-digested pBabehygro. The PCR-derived fragment was confirmed by DNA sequence analysis.

mutations in the N-terminal SH3 domain, the SH2 domain or the C-terminal SH3 domain²⁰, and analogous SH3 mutations in human Grb2 also impair its function²⁶. We therefore used site-directed mutagenesis to introduce these mutations in the SH3 domains of Grb2myc and analysed their effect on mSos1 binding. A proline to leucine substitution at residue 49 (P49L) in the N-terminal SH3 domain of Grb2 decreased its overall binding to mSos1. The mutant bound efficiently to the intact GSTmSos1 Cterminus protein, but binding to the fusion protein containing only residues 613–1,166 was greatly reduced, as was binding to degradation products of the mSos1 C-terminal fusion protein (Fig. 2A, b). The N-terminal SH3 domain of Grb2 thus appears to be responsible for binding to the 31 amino-acid domain of mSos1 described above.

This 31-residue domain of mSos1 contains the sequence VPVPPVPP (single-letter amino-acid code), which differs only slightly from the SH3-binding binding consensus of XPXXPPVXP (where X is any amino acid and Ψ is hydrophobic)³². In contrast to the P49L mutation, a glycine to arginine mutation at codon 203 (G203R) in the C-terminal SH3 domain also decreases overall binding to the mSos1 C terminus, but leaves binding to the 613–1,166 domain intact (Fig. 2A, c). The binding site for the C-terminal SH3 domain must therefore be distinct from that for the N-terminal SH3, and must be located near the C terminus of mSos1. There are several proline-rich stretches in this region that are candidate binding sites. No specific binding by a 49L/203R double mutant of Grb2myc in which both Grb2 SH3 sites have been altered was observed (Fig. 2A, d), indicating that Grb2:mSos1 binding depends upon the cooperative actions of the two Grb2 SH3 domains, either of which can mediate loose binding to mSos1 on its own.

These associations were further confirmed by expressing myc-tagged Grb2 or Sem-5 in Cos1 cells and measuring the ability of the mSos1 C-terminal region immobilized on glutathione agarose beads to absorb these proteins from cell lysates. As seen

in Fig. 2B, these beads were indeed able to bind both Grb2 and Sem-5, while control GST beads showed no affinity for either.

In other experiments, a Grb2 mutant carrying a glutamic acid to lysine substitution at residue 89 in the SH2 domain (again analogous to a loss-of-function *sem-5* allele) also bound to the mSos1 beads efficiently (results not shown). Experiments with Cos cells transiently transfected with SH3 mutants were also consistent with the results described above (data not shown). These data indicate that the proteins Grb2 and Sem-5 share a high affinity for the C-terminal region of mSos1 despite more than 600 million years of separate evolution. High-affinity binding depends on the cooperative binding of the Grb2 N-terminal and C-terminal SH3 domains to a proline-rich motif between mSos1 residues 1,135 and 1,166 and a second site mapping closer to the C terminus of mSos1, respectively.

mSos linked to tyrosine kinase signalling

Grb2 has a second, distinct binding activity that allows it to associate with autophosphorylated tyrosine kinase receptors²⁶.

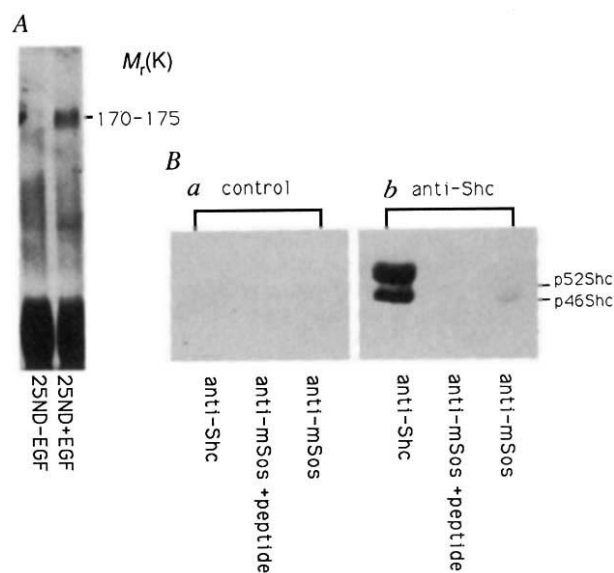


FIG. 3 Association of mSos with activated tyrosine kinase growth factor receptors and the kinase substrate Shc. A, Lysates from NIH/EGFR-25ND cells were either untreated or treated with 100 ng ml^{-1} EGF, immunoprecipitated with anti-mSos antiserum and western blotted with anti-phosphotyrosine monoclonal antibody 4G10. B, Lysates from v-src-transformed Rat2 cells were immunoprecipitated with either anti-Shc serum, anti-mSos peptide serum in the presence of competing peptide, or anti-mSos peptide serum in the absence of competing peptide. The resulting precipitates were probed with either control rabbit serum (a) or with anti-Shc serum (b).

METHODS. A, Two confluent dishes of NIH/EGFR-25ND cells were serum starved for 18 h in 0.2% fetal calf serum. One culture was treated with 100 ng ml^{-1} EGF for 5 min. Cells were washed, lysed as in Fig. 1b, clarified by centrifugation, precleared with protein A-Sepharose, then incubated with anti-mSos peptide serum. (Anti-mSos serum was generated against residues 100–120 of mSos1 and affinity purified, as described in detail elsewhere⁴⁵). Immunoprecipitates were washed as in Fig. 1b, separated on a 7.5% SDS-PAGE gel, transferred and probed with 4G10 anti-phosphotyrosine monoclonal. B, High-density Ratsrc2 cultures were collected in lysis buffer as described in Fig. 1a and clarified by centrifugation. Lysates were precleared with protein A-Sepharose beads and then incubated for 1 h with either precoupled anti-Shc protein A-Sepharose beads⁴¹ (anti-Shc antibodies from UBI, Lake Placid, NY), anti-mSos peptide serum precoupled to protein A-Sepharose beads⁴¹, or anti-mSos peptide serum precoupled to protein A-Sepharose beads that has been preincubated with the immunizing peptide. Beads were washed as in Fig. 2B, eluted in sample buffer for 15 min at 70°C , and then resolved on a 10% SDS-PAGE gel. This gel was then transferred to nitrocellulose, blocked and probed as in Fig. 1a except that the primary antiserum used was either control rabbit serum or anti-Shc antiserum and the secondary antibody used was HRP-conjugated goat anti-rabbit Fc antiserum (Pierce).

This binding is mediated by specific phosphotyrosine-containing peptides on the receptor and the SH2 domain of Grb2 (ref. 26). Taken together with the Grb2:mSos1 association found here, these earlier data indicate that Grb2 may function to recruit mSos1 into a multiprotein complex with an autophosphorylated receptor containing tyrosine kinase by acting as a bridge between the receptor and mSos1. We therefore used an affinity-purified anti-peptide antiserum raised against residues 100–120 of mSos1 and mSos2 (the two are identical in this region) to search for complexes between the endogenous mSos proteins and epidermal growth factor (EGF) receptors overexpressed on the surface of NIH/EGFR-25ND cells³³. This cell line is a derivative of NIH 3T3 transfected with a construct expressing the human EGF receptor³³, and is analogous to the NIH 3T3 derivative HER14 in which the association of Grb2 with activated EGF receptors was demonstrated²⁶. mSos precipitates from EGF-treated cells contained a diffuse 170–175K phosphotyrosine-containing band with the mobility of the EGF receptor which was absent in precipitates from cells that had not been treated with EGF before cell lysis (Fig. 3A). EGF treatment of cells thus results in the formation of multi-protein complexes containing both the EGF receptor and the mSos protein(s).

Another phosphotyrosine-containing protein that can associate with Grb2 is the recently described Shc oncogene product^{34,35}, which is heavily phosphorylated on tyrosine residues in cells transformed by the non-receptor tyrosine kinases v-Src and v-Fps³⁶. Shc can transform fibroblasts and induce PC12 pheochromocytoma cells to differentiate³⁵. The induction of PC12 differentiation is blocked by the N17 dominant inhibitory mutant of Ras³⁵, however, indicating that Shc, like Grb2, functions upstream from Ras. We therefore considered whether Shc acts analogously to autophosphorylated receptors like the EGF-R in binding to mSos by its phosphotyrosine residues.

To explore this possibility, we tested whether Shc is bound to mSos proteins in the Src-transformed Ratsrc2 line³⁷. mSos

precipitates from these cells were resolved by gel electrophoresis and analysed by western blotting with either a polyclonal anti-Shc antiserum (Fig. 3B, b) or with a control rabbit serum (Fig. 3B, a). Both p46Shc and p52Shc were specifically detected in the mSos immunoprecipitates unless the antiserum was preabsorbed with the mSos-derived immunizing peptide (Fig. 3B, b). Shc precipitation by the mSos antiserum must thus have been due to interactions between Shc and mSos in the immunoprecipitates and not to nonspecific crossreactivity of the antiserum with Shc.

The mSos proteins therefore occur in complexes containing a tyrosine kinase receptor (the EGF receptor) or a tyrosine kinase substrate (the Shc protein), both of which also bind Grb2 through its SH2 domain. As the mSos1 protein associates with the SH3 domains of Grb2, Grb2 appears to act as a bridging protein coupling tyrosine kinase receptors or tyrosine kinase substrates like Shc to mSos proteins. The formation of such complexes suggested, in turn, that these represent a means by which tyrosine kinases convey signals to mSos proteins.

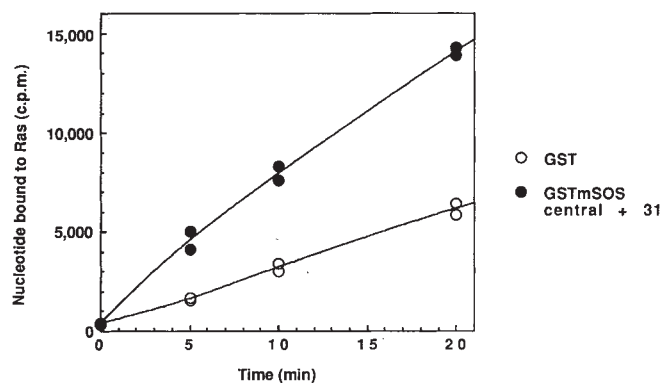


FIG. 4 Guanine-nucleotide-exchange activity of mSos1 towards Ras. [α -³²P]GTP binding to H-Ras was determined in the presence of GST-Sos fusion protein or GST alone.

METHODS. Assays were done in 20 μ l 50 mM HEPES pH 7.5, 1 mM MgCl₂, 1 mM DTT, 100 mM KCl, 0.1 mg ml⁻¹ BSA, 0.2 mM PNPP, 0.05% Triton X-100 and 1.5 μ M [α -³²P]GTP (2.5 μ Ci) with 1 μ g fusion protein. This GSTmSOS1 fusion protein was isolated from soluble lysates of induced bacteria expressing the GSTmSOS1central + 31 fusion protein as described in Fig. 2. About 2% of the fusion protein represented full-length product. The remaining 98% of the soluble fusion protein is a C-terminal breakdown product of about 28K which encodes little more than the GST peptide. Reactions therefore included about 20 ng of the GSTmSOS fusion protein. The reaction was started by addition of 10 ng, per assay point, of Ras. After the indicated time, samples were put on ice and binding stopped by addition of 1 ml 50 mM HEPES pH 7.5, 5 mM MgCl₂, 1 mM DTT, 10 μ l ml⁻¹ BSA, 0.1 mM unlabelled GTP. Supernatant (900 μ l) was then spotted onto nitrocellulose filters, washed three times with 5 ml cold PBS, 5 mM MgCl₂, and the radioactivity bound to the filters was Cerenkov-counted.

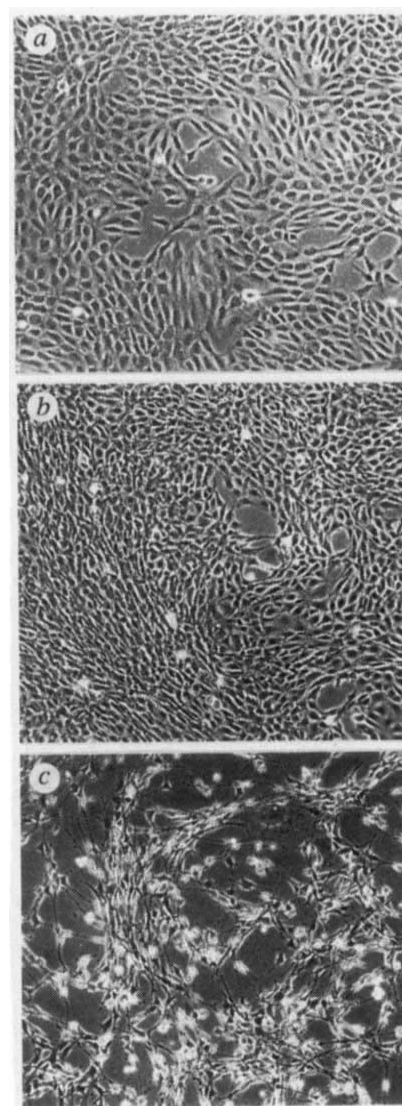


FIG. 5 Morphology of parental Rat1 fibroblasts (a) and DSos-infected derivative cell lines Rat1.SosF7C (b) and Rat1.SosF10C (c).

METHODS. Rat1 cells were infected with viral supernatants from a cloned Ω E cell line transfected with pBabeHSos as described above. Transformed foci were isolated by micromanipulation and grown into stable cell lines.

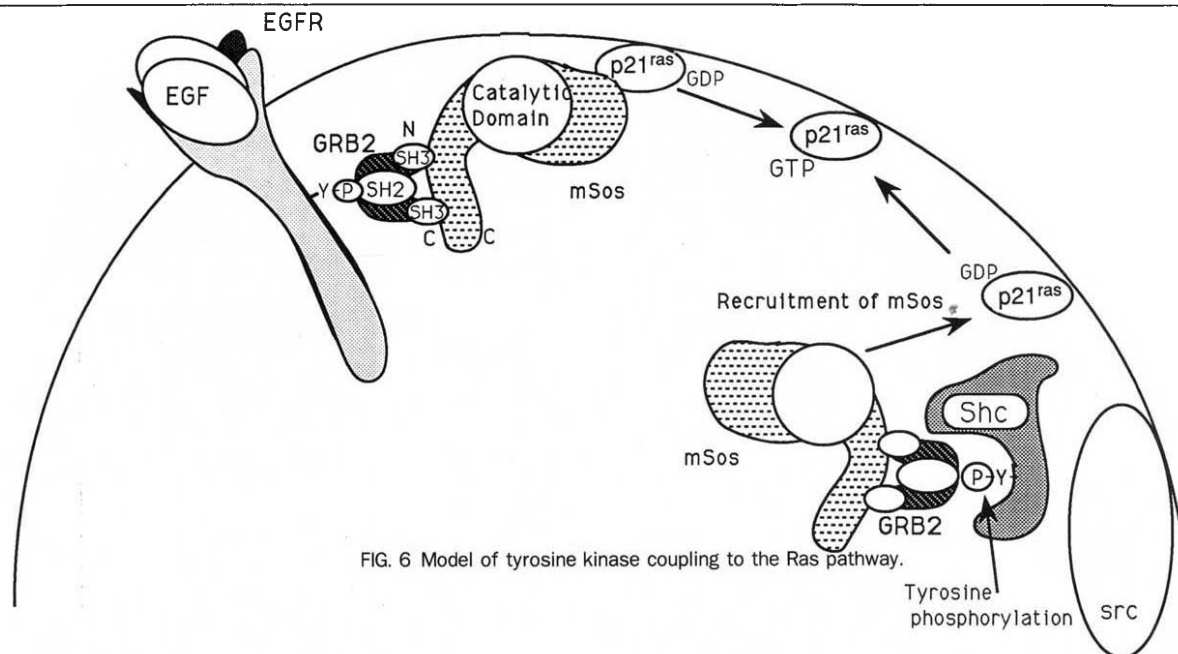


FIG. 6 Model of tyrosine kinase coupling to the Ras pathway.

mSos1 is a Ras nucleotide-exchange protein

Sos proteins are homologous to several yeast nucleotide exchange factors that induce guanine nucleotide exchange by yeast Ras proteins^{13,14,28}. mSos1 has not, however, been shown to induce nucleotide exchange by mammalian Ras.

Bacterially expressed GST-Sos fusion protein containing residues 613–1,166 of mSos1, was therefore purified and tested for its ability to increase the rate at which guanine nucleotide binds to H-Ras. The rate at which [α -³²P]GTP was loaded on H-Ras was three times higher in the presence of the Sos fusion protein than in the presence of GST control showing that mSos can act as a guanine nucleotide exchange factor for Ras *in vitro* (Fig. 4). Additional experiments revealed that the mSos1 catalytic-domain fusion protein also stimulated release of guanine nucleotides from the Ras protein (data not shown). This Sos fusion protein had no exchange activity towards the closely related protein R-Ras (data not shown), indicating that the Sos–Ras interaction was highly specific. By promoting physical complexes between tyrosine kinases (or their substrates) and the mSos proteins, Grb2 thus couples such kinases to a protein (mSos) capable of activating the Ras signalling pathway.

Morphological transformation by *Drosophila* Sos

Mutation or overexpression of the components of this signal transduction pathway, including growth factors and their tyrosine kinase receptors, nonreceptor tyrosine kinases and their substrate Shc, Grb2 and Ras, results in the induction of cell growth or transformation. The only components not so far shown to do so are the Sos proteins. We therefore devised a test for Sos function to strengthen the conclusion that the Sos proteins participate in this cascade.

As this pathway has apparently been strongly conserved in evolution, we reasoned that the *Drosophila* Sos protein might couple tyrosine kinases to Ras in mammalian cells and that its overexpression there should stimulate the Ras pathway. As an assay for biological function, we scored the appearance of morphologically transformed cell foci in monolayers of rat cells exposed to a retrovirus transducing the *Drosophila* Sos gene (DSos). As a positive control, we used a retrovirus transducing an oncogenic *neu* allele. Helper-free retroviral stocks were generated from cloned producer lines expressing DSos/hygro virus, Neu/hygro virus or hygro virus, and transforming potential was measured as the ratio of transformed foci to hygromycin-resis-

tant colonies for each viral stock. Rat1 fibroblast cells were used for these assays, as they have a low rate of spontaneous transformation and, unlike NIH 3T3 cells, are not sensitive to weakly oncogenic stimuli like those resulting from overexpression of normal Ras³⁸.

The DSos virus induced focal transformation of these cells, but with less than one-tenth the efficiency of the *neu* virus (Table 1). In addition, the *neu*-induced foci typically appeared earlier than DSos foci. Weak transformation of NIH 3T3 cells by the catalytic domain of a yeast protein that can stimulate the exchange of guanine nucleotides on Ras has been previously reported³⁹.

Foci were picked to establish stable DSos-infected cell lines. These lines were found to express DSos RNA on northern blots (data not shown), and were refractile, growing to a high saturation density (Fig. 5). The *Drosophila* Sos protein, when overexpressed in mammalian cells, is thus able to trigger growth-stimulating pathways, such as the Ras pathway, so as to produce growth deregulation and transformation. This strengthens the idea that Sos proteins, initially discovered in the context of differentiating fly eye tissue, are so well conserved in evolution that they can participate in activating a mammalian mitogenic pathway.

Conclusions

An extensive body of data indicates that receptor and nonreceptor tyrosine kinases lie upstream of Ras in a signalling cascade that is widely conserved among metazoa. This signalling cascade is used to transduce both mitogenic and differentiation signals and seems to operate in some form in virtually all cell types studied to date. In spite of this intensive study, the biochemical links in this cascade have until recently remained unclear.

We have shown here that several of the components of this cascade are able to form physical complexes with one another. The central protein in this interaction is Grb2. On the one hand, it can bind tightly to mSos1 through its two SH3 groups; on the other, it can bind to tyrosine kinase substrates^{26,55} (in this case either the autophosphorylated tail of the EGF receptor, or Shc, a substrate of the Src kinase) through its SH2 group. This bifunctionality allows it to serve as an adapter molecule that brings together the upstream signalers (the kinases) and the element two steps further down the cascade (mSos).

The mSos proteins are homologous to several yeast factors that stimulate GTP loading onto Ras. Such loading activates

these Ras proteins, allowing them to transmit signals to subsequent steps in the pathway. Here we have shown that mSos1 too can increase GTP loading on mammalian Ras, and that overexpression of the *Drosophila* mSos homologue, *DSos*, can transform rat cells.

Together, these experiments solidify thinking about the organization of the tyrosine kinase to Ras signalling cascade, which can now be schematized as depicted in Fig. 6.

Nonetheless, vital pieces of evidence supporting this scheme are still lacking. Although Grb2 has all of the attributes of the adaptor that links an autophosphorylated receptor to mSos, and such complexes can indeed be observed in the living cell, we have not yet succeeded in showing that the formation of the observed (EGF) receptor:mSos complexes depends on Grb2 rather than another bridging molecule with an

analogous function.

The functional significance of the observed, multi-protein complexes also needs further clarification. Although there is strong evidence that these complexes assemble in the presence of mitogens, we have not shown directly that their formation is tightly linked with the transduction of signals between tyrosine kinases, their substrates and mSos1. But loss-of-function Grb2 mutants with altered SH3 domains are unable to induce DNA synthesis in cooperation with Ras²⁶, and have altered mSos1 binding profiles, indicating that mSos1:Grb2 complex formation and induction of DNA synthesis by Grb2 are indeed functionally linked. The signal transduction cascade characterized in this report is likely to represent only one of several ways in which tyrosine kinases affect Ras function and thereby trigger cell growth and differentiation. □

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LETTERS TO NATURE

Hot gas and dark matter in a compact galaxy group

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COMPACT groups of galaxies have such high apparent densities that their members are separated in projection by only a few galactic radii, making them the most promising environment in the low-redshift Universe for studying the effects of galaxy merging. But the dynamical and evolutionary status of these groups is still subject to considerable uncertainty^{1–3}, partly because of the bias introduced by the selection criteria used to identify them⁴. Using the Rosat X-ray telescope⁵, we have detected a large cloud of hot gas centred on the compact group HCG62. From the properties of this gas, rather than the galaxies themselves, we are able to infer both the distribution of dark matter (which extends significantly beyond the apparent optical confines of the group and dominates its total mass) and the evolution of the group as a whole. The compact core of galaxies seems to have formed as a result of orbital decay in a much more extended system, and should culminate in a final merger within a few billion years. We suggest

that a new class of 'fossil' groups, consisting of a large elliptical galaxy embedded at the centre of an extended X-ray halo, awaits discovery by Rosat.

We observed the compact group HCG62, from Hickson's compilation⁶, in December 1991. In the 19,724-s exposure, the position-sensitive proportional counter (PSPC) on Rosat detected more than 9,000 counts from an extended source centred on the galaxy group (Fig. 1). The four catalogued galaxies, marked *a–d* in the figure, have extinction-corrected blue magnitudes of 13.4, 13.8, 14.6 and 15.8 respectively, and are classified⁷ as E or S0.

A radial profile of the diffuse X-ray flux about its centroid (Fig. 2) shows that the emission can be traced out beyond the PSPC window support ring, which interrupts the data at a radius of $r \approx 20'$. A core-index model of the form generally used to fit galaxy clusters⁸

$$S(r) = S(0)[1 + (r/a)^2]^{-\alpha}$$

gives a reasonable fit at $r \geq 4'$, but a second component is required to model the rise in surface brightness near the centre. The low-energy spectrum is substantially affected by uncertainties in background subtraction (see Fig. 1), so we restrict our spectral work to the range 0.5–2.3 keV and add a 10% background systematic in quadrature to the errors arising from counting statistics. We also allow for the redshift, $z = 0.0138$, and

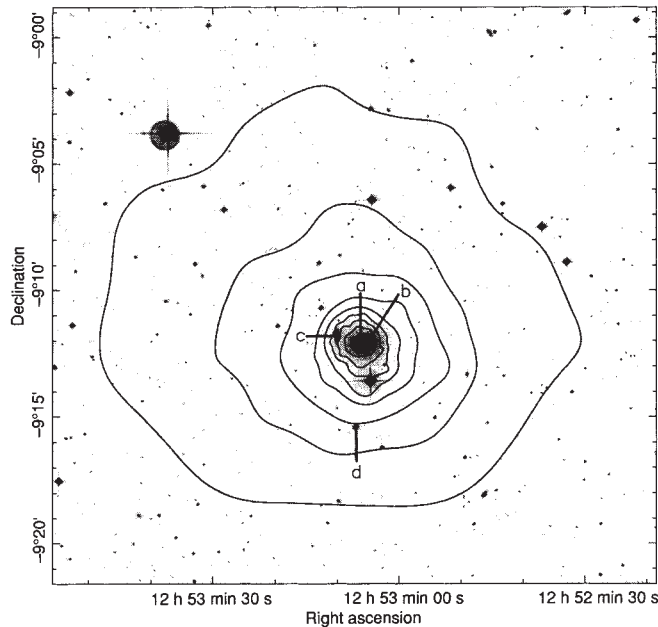


FIG. 1 Contours of X-ray surface brightness in the 0.6–2.3 keV band (chosen to optimize signal to noise ratio) are overlaid on a UK Schmidt blue image of the field, with the four HCG62 galaxies marked. Point sources have been removed from the X-ray image and an estimate of the background flux subtracted. Contours are separated by factors of 2, and have been smoothed increasingly heavily at low surface brightness to suppress noise. The X-ray emission appears smooth and regular. In the inner region it is extended north-south and centred, within the 10'' attitude errors, on the largest elliptical galaxy in the group, HCG62a, which may be interacting with the adjacent galaxy HCG62b. No X-ray flux is detected from any individual galaxy, a result consistent with normal X-ray/optical flux ratios for early type galaxies²⁵. The background is derived by recording a spectrum in source-free regions of an annulus at $r=0.7\text{--}0.8^\circ$, estimating the particle contribution from the master veto rate²⁶ and extrapolating the remainder using the energy-dependent vignetting function, to give a model for the spectral background throughout the field. Tests on a set of deep pointings containing no bright sources indicate that this background estimate is good to $\sim 10\%$.

adopt a corresponding distance to the system of $83h_{50}^{-1}$ Mpc, where $h_{50} \equiv H_0/50 \text{ km s}^{-1} \text{ Mpc}^{-1}$.

The integrated source spectrum within $r=15'$ is well fitted ($\chi^2=9.0$ with 15 degrees of freedom) by a Raymond and Smith⁹ (RS) hot plasma model with temperature $T=0.96 \pm 0.04$ keV and metallicity $Z=0.15 \pm 0.06$ solar (1 sigma errors), where the absorbing column density has been fixed at $n_H=3.0 \times 10^{20} \text{ cm}^{-2}$ as derived from radio surveys¹⁰ (both T and Z are insensitive to the precise n_H value adopted). The bolometric luminosity is $L_X=1.1 \times 10^{43} h_{50}^{-2} \text{ erg s}^{-1}$.

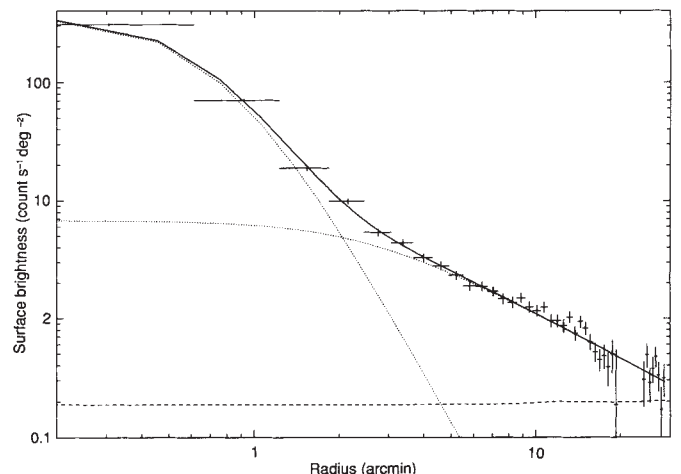
The spectral hardness ratio varies substantially with radius. RS fits to spectra extracted from annuli of width $2.4'$ about the centroid show (Fig. 3) that the temperature dips sharply in the centre, peaks at $r \approx 3'$ and drops steadily at larger radii. The metallicity is less well defined, but it is clear that it is modest: $Z \approx 0.3$ is consistent with all the data, and $Z < 0.6$ at 90% confidence for annuli within $r=7.2'$. This contrasts with the high metallicity ($Z > 1$) expected in gas ejected from galaxies, and suggests that the intragroup medium is predominantly primordial gas, with some galactic gas intermixed.

The surface brightness profile of Fig. 2 can be deprojected, under the assumption of spherical symmetry, to give emissivity in counts per second per unit volume, and hence, using temperatures from Fig. 3, the electron density n_e . In the central region ($r \approx 0.5'$) this gives $n_e \approx 0.01 \text{ cm}^{-3}$. For $T_0 \approx 0.8$ keV this

implies a cooling time $t_{\text{cool}} = 9 \times 10^8 h_{50}^{-1/2} \text{ yr}$. This short time strongly suggests that the central cool, high-surface-brightness component is a cooling flow. The alternative, that it is a cloud of cooler gas ejected from the central galaxies, seems unlikely given its low metallicity. Both Fig. 2 and Fig. 3 suggest that the outer limit of this component, which corresponds to the radius at which cooling becomes significant, lies at $r_{\text{cool}} \approx 2.5'$. The cooling time at this point, $\sim 1 \times 10^{10} \text{ yr}$, indicates that the system must be several billion years old.

To investigate the distribution of gas outside r_{cool} , we adopt the model fitting approach used previously for galaxy clusters¹¹. The gas density is parameterized as $n_e(r) = n_e(0)[1 + (r/a)^2]^{-\alpha}$, and the temperature as a simple ramp, $T(r) = T(0) - r(dT/dr)$. The emission from this gas is then computed using an RS model with $Z=0.3$, together with $3 \times 10^{20} \text{ cm}^{-2}$ of interstellar absorption, and is projected along the line of sight, blurred with the point spread function and folded through the PSPC energy response, allowing for the redshift and taking $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. This generates a predicted dataset which is compared with the observed 0.5–2.3 keV spectral image from $r=3'$ to $12'$. As the data are strongly poissonian, the model parameters are adjusted to maximize the likelihood function, rather than minimizing χ^2 . The best-fit model has $\alpha=0.54$ and a small core radius $a \approx 0.5'$, so that at $r \geq 3'$, where the model applies, the density is $n_e(r) = 3.0 \times 10^{-3} r^{-1.08} \text{ cm}^{-3}$, where r is

FIG. 2 Radial profile of the 0.6–2.3 keV image (corrected for background and vignetting) about the X-ray centroid. A two-component core-index model (see text) is overlaid, and gives a reasonable representation of this profile: the outer component has a core radius $a=2.5'$ and $\alpha=0.64$, and the central enhancement has $a=0.8'$, $\alpha=2.2$. For comparison, the point spread function of the PSPC has a half-energy radius of $0.25'$. The level at which systematic errors in background subtraction may be present is indicated by the dashed line. The gap in the data at $r \approx 20'$ is caused by the PSPC window support ring.



in arcminutes. The temperature drops from 1.3 keV at $r=3'$ to 1.0 keV at $r=14'$, a decline which is significant at 95% confidence.

Although this model has been fitted to data only out to $r=12'$, the continuity in surface brightness apparent in Fig. 2 suggests that it may reasonably be extrapolated at least as far as the PSPC ring ($r=20' \approx 0.48 h_{50}^{-1}$ Mpc). The gas mass within this radius is $M_{\text{gas}} = 2.5 \times 10^{12} h_{50}^{-2.5} M_{\odot}$. The total blue luminosity of the four galaxies in HCG 62 is $L_B = 9.5 \times 10^{10} h_{50}^{-2} L_{\odot}$. Hence, assuming a galaxy mass/light ratio¹² $M/L_B = 8(M_{\odot}/L_{\odot})$, the visible mass in galaxies is $M_* = 8 \times 10^{11} h_{50}^{-1} M_{\odot}$, and the ratio of gas to stellar mass within $r=20'$ is $M_{\text{gas}}/M_* = 3.1 h_{50}^{-1.5}$, a value at least as high as is seen in most galaxy clusters^{13,14}.

Given $n_e(r)$ and $T(r)$, together with the assumption that the gas is in hydrostatic equilibrium in the potential well of the group (which seems reasonable given the regular X-ray morphology), the gravitating mass within radius r can be derived using the equation

$$M_g(<r) = -\frac{rkT(r)}{G\mu m_p} \left[\frac{d \ln n_e}{d \ln r} + \frac{d \ln T}{d \ln r} \right]$$

where $\mu \approx 0.6$ is the mean molecular weight in the ionized plasma and m_p is the proton mass. The resulting M_g profile rises very nearly linearly, $M_g \approx 1.3 \times 10^{12} r h_{50}^{-1} M_{\odot}$ (r in arcmin), so that $\rho_g \propto r^{-2}$; that is, the gravitating mass is distributed as an isothermal sphere. Because its mass diverges, this distribution must cut off at some radius $R_t \geq 20'$. The mass/light ratio for the system is then $M_g/L_B = 270(R_t/20') h_{50} M_{\odot}/L_{\odot}$, which is at least as large as in galaxy clusters^{11,15,16}. Most of this mass is dark, the fraction in observable baryons (for $h_{50}=1$) being

$$f_{\text{baryon}} = \frac{M_* + M_{\text{gas}}}{M_g} = 0.03 \left(\frac{R_t}{20'} \right)^{-1} + 0.10 \left(\frac{R_t}{20'} \right)^{0.92} \geq 0.13$$

Comparing ρ_g to an isothermal sphere model¹⁷ gives a one-dimensional velocity dispersion for the dark matter of $\sigma_g = 343 \text{ km s}^{-1}$, very similar to the observed velocity dispersion¹⁸ (331 km s^{-1}) of the galaxies. The characteristic 'virial radius' of the mass distribution defined by

$$R_v = \left(\sum_i m_i \right)^2 / \sum_{j < i} \frac{m_i m_j}{r_{ij}}$$

is useful for comparison with the results of N -body simulations. Applying this formula to the ρ_g distribution, one finds $R_v = R_t \geq 20'$, whereas for the optical galaxies (using luminosity in place of mass in the equation, and allowing for the effects of projection in the standard way) one obtains a value $R_v = 3.6'$; that is, the optical size of the group gives a gross underestimate of the extent of the gravitating matter. The total mass of a stable,

gravitationally bound system is commonly estimated from its size and velocity dispersion using the expression for the 'virial mass', $M_v = 3\sigma^2 R_v / G$. In the present case, the galaxy distribution gives $M_v = 7 \times 10^{12} h_{50}^{-1} M_{\odot}$, which underestimates the mass by a factor $3.7(R_t/20')$.

These results compare well with the simulations of Barnes¹⁹, who found that the optical galaxies in an evolving group with a dark matter background congregate progressively towards the centre, because of the effects of dynamical friction and mergers. This reduces R_v whilst σ is largely unchanged, so that M_v underestimates the total group mass by a factor ~ 3 . The timescale on which galaxy merging proceeds is $t_v = R_v / \sqrt{3}\sigma^2$, which drops as R_v shrinks. Hence the current value of t_v is a measure of its remaining lifetime (which is a few times t_v), rather than its age, which can be many times larger. For HCG 62, $t_v = 1.6 \times 10^8 h_{50}^{-1} \text{ yr}$, but the initial evolutionary timescale (based on the ρ_g distribution) was larger by a factor $R_t/3.6'$.

Dynamical friction¹⁷ causes a galaxy moving at a typical velocity $\sqrt{3}\sigma = 574 \text{ km s}^{-1}$ to lose energy to the dark matter background on a timescale of

$$t_{\text{fric}} \equiv -v/\dot{v} \approx \frac{3 \times 10^9}{\rho_{-2} M_{12}} \text{ yr}$$

where M_{12} is the galaxy mass in units of $10^{12} M_{\odot}$, and ρ_{-2} is the density of the medium through which it is travelling, in units of $10^{-2} \text{ a.m.u. cm}^{-3}$. For HCG62a, this timescale at $r = 250 h_{50}^{-1} \text{ kpc}$ is $\sim 2 \times 10^9 \text{ yr}$ if it has a massive dark halo, and $2 \times 10^{10} \text{ yr}$ if it does not. Hence dynamical friction can account for substantial decay of galaxy orbits provided the group is several billion years old, and the galaxies started with dark haloes. Mamon¹ found in simulations of the evolution of loose groups that the effects of orbital decay were too weak to account for the formation of bound cores of galaxies (the galaxies generally merged before a group of 3–4 could be collected) and concluded that most apparently compact configurations must be chance alignments within looser groups. However, he started with a much less substantial dark matter background ($M/L \approx 50 M_{\odot}/L_{\odot}$) than we see in HCG62.

It seems that the properties of HCG62 can be understood in terms of a model in which the system separated out of the Hubble flow and virialized several billion years ago. The gravitating mass formed a roughly isothermal distribution, truncated at a radius $R_t \geq 0.5 h_{50}^{-1} \text{ Mpc}$, and would be classified as a loose group. A substantial ($M_{\text{gas}} > M_*$) primordial intragroup medium (IGM) was present from the outset, and its peak temperature is readily explained by compression within the potential well. In its inner regions ($r < 75 h_{50}^{-1} \text{ kpc}$) this IGM is dense enough to have cooled significantly over the age of the system, and gas is currently cooling out at a rate $\sim 10 M_{\odot} \text{ yr}^{-1}$. The galaxies initially had massive dark haloes (and hence $M/L_B \sim 80 M_{\odot}/L_{\odot}$), and several of them lost energy by dynamical friction and settled towards the centre of the group where they are now found. These galaxies are all of type E or S0, but are not unusually blue⁷, and so cannot have formed³ by spiral merging within the last $\sim 2 \text{ Gyr}$; either their type is primordial, or they were generated by mergers early in the life of the group. Gas has been ejected by these galaxies during their evolution, but this probably constitutes only 10–30% of the IGM, and is fairly well mixed out to $r \geq 250 h_{50}^{-1} \text{ kpc}$.

There may still be additional galaxies bound to the group at larger radii whose orbits have not decayed greatly. Hickson's isolation criterion⁶ requires that there should be no further bright galaxies within $r \approx 6'$, but two studies^{20,21} of the environments of compact groups find evidence for enhanced galaxy density within $r = 20\text{--}30'$ in HCG62. It is therefore possible that the estimates of L_B and hence M_* used above are rather low. Correcting for this would bring M/L_B and M_{gas}/M_* into better agreement with typical values for clusters.

N -body simulations indicate that HCG62 will evolve rapidly

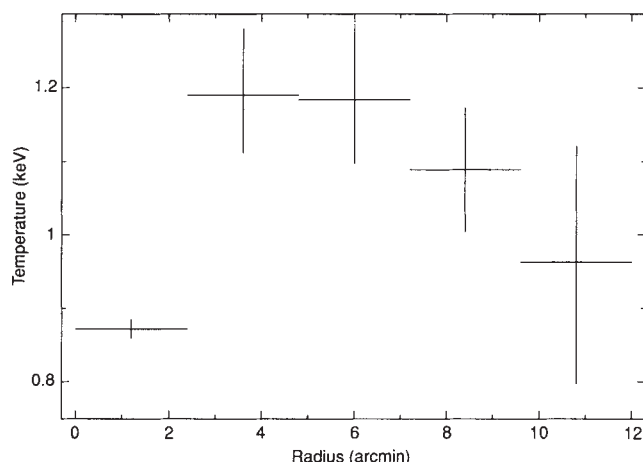


FIG. 3 Temperature from RS model fits to spectra from successive 2.4' rings about the X-ray centroid. Errors are 1 sigma.

in the future. If the central galaxies have retained their original dark haloes they will merge on a timescale $\sim t_V$, and within ~ 1 Gyr will probably have formed a single large elliptical remnant²². If the haloes have been stripped, then the merging process will take several times longer^{1,19}. The rather low incidence of observed merging within compact groups³ supports the second option. When merging is completed, the remnant will be embedded at the centre of the largely dark remains of the group, and will retain its present extended, luminous X-ray halo. Systems such as this will be readily distinguishable from normal elliptical galaxies in the X-ray, and their space density should be comparable to that²² of compact groups ($1-2 \times 10^{-6} \text{ Mpc}^{-3}$). Many such 'fossil groups' should therefore be detected by Rosat, providing a valuable sample of merger-ellipticals whose properties can be compared with those of elliptical galaxies in general.

After completion of this work we became aware of the Rosat study^{23,24} of the NGC2300 galaxy group. Our results are similar in several respects: the groups have similar mean gas temperatures and low metallicities, and are dominated by dark matter, but HCG62 contains considerably more gas. Mulchaey *et al.* note that the low baryon fraction they deduce for their group is consistent with the fraction ($f_{\text{baryon}} = 0.04-0.08$) expected in a critical-density ($\Omega = 1$) Universe. The baryon fraction is determined more reliably in HCG62, as we have been able to map the gas temperature, and we find $f_{\text{baryon}} \geq 0.13$. The density profile of the gas is flatter than that of the dark matter, so f_{baryon} increases with radius, and it seems unlikely that its

value within any particular radius can be regarded as representative of the Universe as a whole. $\square$

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Explosive ejection of matter associated with star formation in the Orion nebula

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TIGHTLY collimated outflows of gas are often associated with regions of star formation; they interact with the ambient interstellar medium to produce the knots of shock-excited emission known as Herbig-Haro (HH) objects^{1,2}. Two interpretations have been suggested for these objects: they may represent either the shocking of dense clumps of material that have been ejected into the surrounding molecular cloud³, or the interaction of stationary knots with fast, low-density jets^{4,5}. Here we report the discovery of a complex of HH objects and associated wakes that require compact knots of material to have been ejected over a wide opening angle in a seemingly explosive event. Our observations suggest that, at least in this case, the former interpretation is correct, and they highlight the need to search other star-forming regions in order to establish the frequency of such events.

The main star-forming region of the Orion nebula, OMC-1, contains several luminous young infrared sources, primarily the Becklin-Neugebauer object (BN)⁶ and the younger, more luminous IRC2⁷. Shocked molecular hydrogen occupies two broad fans that suggest bipolar ejection with a wide opening angle⁸. The northern fan contains linear features⁹ seemingly radiating from the lower-luminosity source IRC9. At least seven HH objects in the same region have been revealed by emission of [O I] at a wavelength of 630 nm (refs 10, 11); they have linewidths up to 400 km s⁻¹. No connection has previously been made between the linear H₂ features and the HH objects.

Using the infrared camera IRIS on the 3.9-m Anglo-Australian Telescope, we have imaged the region through narrow-band filters selecting the [Fe II] $a^4F_{5/2} - a^4D_{7/2}$ line at 1.644 μm and the 2.122- μm $v = 1 \rightarrow 0$ S(1) transition of H₂. Both are sensitive

tracers of shock activity and suffer little from the high extinction experienced at optical wavelengths. Figures 1 and 2 show the images in the two lines. To our knowledge, this is the highest-spatial-resolution image of OMC-1 in H₂ yet obtained, and the first in [Fe II]. In the northern lobe (Fig. 1) we resolve the H₂ into at least 20 hollow structures closely resembling classic bow shocks. Most have open tips. Compact [Fe II] emission knots coincide with the tips of most H₂ bows. All [O I] knots have corresponding [Fe II] features.

Outflow seems to be common in young stars approaching the main sequence¹². The shocking of the HH objects indicates that this outflow is tightly collimated, and this is usually attributed to confinement by a gas disk or a wind emanating from an accretion disk (see, for example, ref. 13).

In discussing their [O I] observations, Axon and Taylor^{10,11} favoured the idea that a stellar wind interacted with condensations of ambient material, despite formidable energy requirements. They cited two arguments against ejection: the velocity range of the [O I], and the long-term stability of the knots. Recent models of bow shocks¹⁴ negate the first argument, reproducing well the available velocity profiles which arise in the bow shock and not in the clump as they assumed. We see no problem with stability. Internal expansion can proceed no faster than the sound speed. With Mach speeds of ~ 400 the knots could have expanded only to the resolution limit of our data.

A single precessing jet cannot simultaneously excite all the features, whose cooling times are ~ 1 yr. A series of jets is also unlikely. Although single jets are commonly associated with HH objects, their morphology is quite different from those observed here: they display knots along their length and a more open, often knotted, head¹⁵. Moreover, conservation of momentum flux between material in a jet and a HH knot at its head would require unrealistically high wind speeds, exceeding 1,000 km s⁻¹.

Emission from [Fe II] at 1.644 μm traces shocks with velocities of a few hundred kilometres per second, as in supernova remnants¹⁶. H₂ is dissociated at shock velocities above 25-45 km s⁻¹, so it traces slower or oblique shocks^{17,18}. The interpretation of these images thus seems unambiguous: at least a score of individual knots of gas have been ejected into the surrounding molecular cloud. [Fe II] emission is located within or at the

surface of the knots, where they strike the dense material, whereas H_2 is excited in bow, rather than planar shocks, in their wakes. Our images provide strong morphological support for the ejection model³ of these HH objects. The two brightest [Fe II] knots lack H_2 wakes. We infer that these knots left the densest portions of the molecular cloud, probably with a significant component of velocity towards us. Their brightness may be due to reduced extinction. Detailed images of several of the HH knots in the radiation of [S II], recently made from Hubble Space Telescope (HST) data¹⁹, show them to have complex structures.

Within each knot-wake association the projected extent of the [Fe II] region is $\leq 2 \times 10^{16}$ cm. H_2 emission starts immediately behind the head, perhaps where overpressure from the external medium on the cavity created by passage of the knot pinches the gas. It extends typically $1-2 \times 10^{17}$ cm, to the point where the transverse velocity component can no longer excite H_2 , $\sim 5 \text{ km s}^{-1}$. The width of each bow is about one-third of its length at this point. The thickness of the shock front must be $< 1 \text{ arcsec} \approx 10^{16}$ cm.

By projecting back the axes of symmetry of the H_2 wakes, and faint tails associated with some [Fe II] knots, we find an ejection centre within 5 arcsec of BN and IRc2: either may be the origin. IRc9 is precluded; it was earlier implicated because the chance alignment of a number of short H_2 wakes suggested connected linear features. The opening angle of the ejection is $\sim 90^\circ$.

The bright nebulosity to the south of OMC-1 hampers a study of the southern lobe, covered in Fig. 2. Few similar knots or wakes exist, perhaps because they have been ejected into the ionized cavity created by the Trapezium stars, where low density and high ionization would prevent formation of both emission lines. Structures that might be wakes have been revealed through

HST imaging²⁰ in [O III] and $H\alpha$. Two [Fe II] knots, marked in Fig. 2 and identified^{21,22} as HH 3 and HH 4, have emission tails suggesting an origin in the BN-IRc2 region. These may represent the southern lobe of bipolar ejection. Their projected distance from IRc2 is 0.5 pc; this is $\sim 50\%$ greater than that of the most northerly knot, possibly indicating greater deceleration in the latter.

Spectroscopy of the knots will be described elsewhere. Here we use low-resolution spectra of several, taken with IRIS, to deduce the gas density in the knots. No H_2 emission is seen, as expected. The spectra are typical of collisionally excited [Fe II], and yield electron densities of $\sim 10^4 \text{ cm}^{-3}$. To relate this to the density of material in the knot requires knowledge of the shock structure. We refer to the schematic diagram in ref. 4. The density of 10^4 cm^{-3} refers to swept-up, shocked cloud material. The density in the knot must be at least as large as the shocked cloud material, otherwise the knot would have swept up more than its own mass and have been considerably decelerated. We argue that in fact they have similar densities. The pressure at the contact discontinuity between the shocked cloud material and the knot drives a shock through the knot, so that shocked gas in the knot and the cloud are in pressure equilibrium. We expect the gas to have cooled to comparable temperatures in both regions, so they must have similar densities.

Taking 10^4 cm^{-3} as representative, we derive knot masses by two means. First, the integrated line intensity of a few times $10^{-13} \text{ erg s}^{-1} \text{ cm}^{-2}$ implies that a few times 10^{46} Fe^+ ions are present in each. Our spectra indicate fairly small corrections for extinction. If we assume that the mass fraction of iron has the solar or cosmic value, and that ionization states other than Fe^+ account for 50% of the iron (this figure is compatible with the ionization state shown by other species), then we find knot masses of $\sim 10^{-6}$ solar masses ($M_\odot$). Second, we derive a volume

FIG. 1 Mosaics of 16 IRIS frames of the northern outflow from OMC-1. Left panel, in H_2 ; right panel, in [Fe II]. The image scale is 0.26 arcsec per pixel and the seeing full width at half maximum was about 0.5 arcsec. Much of the diffuse nebulosity in [Fe II] is due to the 12-4 transition of H_I which also lies within the filter bandpass. The coordinate system is 1950. Alongside [Fe II] knots we show the Herbig-Haro numbers of ref. 10. IRc2 lies off the lower edge of this image at (1950) 5 h 32 min 47.0 s $-5^\circ 24' 14''$.

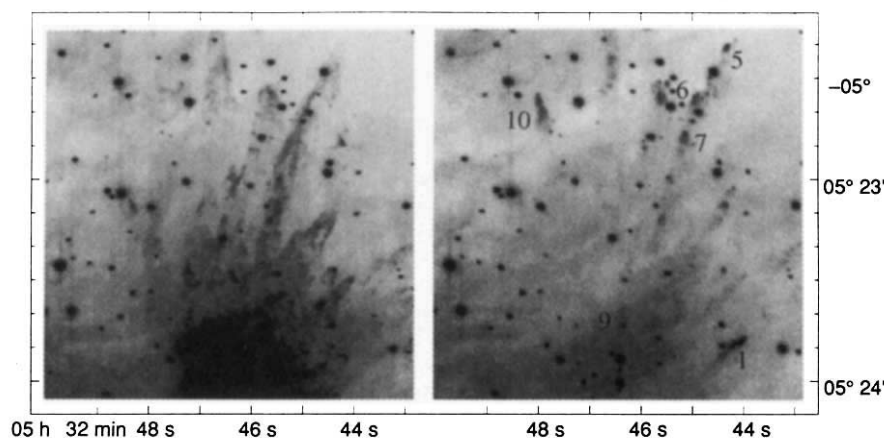
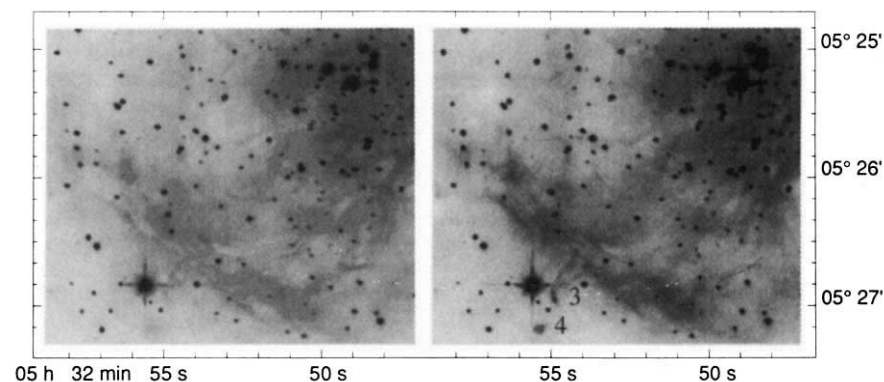


FIG. 2 Portion of mosaics of IRIS frames that show the region southeast of the OMC-1 outflow. The image scale is 0.61 arcsec per pixel and the seeing was < 1 arcsec. The Trapezium stars lie at upper right, and the HH numbers are from ref. 21.



from the apparent size of the knots which for this electron density yields a mass about 1.5 orders of magnitude larger. Both figures should be raised. Iron will be depleted in the gas phase because it is preferentially incorporated in the dust grains within the molecular cloud²³. If the grains are not decomposed in the shock, the mass derived by the first technique may be raised one or two orders of magnitude. Additionally, the core of each knot may not emit [Fe II]. We thus regard $10^{-5} M_{\odot}$ as a minimum mass.

Using the velocities of ref. 10, the present kinetic energy of all the knots is about 10^{44} erg, and initially would have been greater because the knots must have decelerated since ejection. It represents the luminous output of IRC2 (5×10^4 solar luminosity, $L_{\odot}$) over at least a week²⁴. This is large by comparison with most HH objects, but only 10^{-3} of the kinetic energy of the CO-emitting gas¹².

Only a small fraction of the emitting H_2 comes from the well-defined bow shocks in our images. The kinetic energy in the knots is only an order of magnitude greater than the internal energy stored in hot H_2 ($100 L_{\odot}$, cooling in one year²⁵), so cannot provide the energy source for the bulk of the H_2 emission. However, we cannot tell from our images whether the brighter H_2 emission (bottom of Fig. 1) comprises many overlapping bows; if it does the knots there must be slower-moving to produce no [Fe II] emission. Hence we cannot rule out a wind-driven shock to maintain the bulk of the hot H_2 .

The outermost knots require less than 1,000 yr to traverse the distance from the centre of ejection. If deceleration occurs the time will be reduced. As the lifetime of stars like IRC2 is $\sim 10^7$ yr, we are privileged to see this phenomenon, and expect it to be rare elsewhere (although more evolved examples may be identified). We cannot say over what period the ejection took place. The images suggest an explosive event, ejecting debris with a range of velocities.

A possible origin for the ejection is an FU Orionis-type (Fuor) eruption. Fuors are young stars undergoing decade-long increases in luminosity, attributed to accretion disk instabilities²⁶. Each event liberates 10^{45-46} erg of radiant energy. A conversion efficiency to kinetic energy approaching 100% would thus be required to produce the ejection in OMC-1, but we may speculate that scaled-up events accompany massive star formation.

A supernova offers an alternative explanation. Occurring deep within the molecular cloud, its evolution and characteristics would differ from typical remnants. Most of the expansion energy would be quickly radiated through infrared atomic fine structure and molecular cooling lines. A supernova model was considered by Chevalier²⁷ to explain the broad H_2 line emission²⁸ in OMC-1. He postulated that H_2 emission arose from many fast ($\sim 100 \text{ km s}^{-1}$), dense ($\sim 3 \times 10^7 \text{ cm}^{-3}$) knots, but rejected the model after estimating that the bulk momentum in the CO-emitting gas¹² was at least an order of magnitude less than the $\sim 10^4 M_{\odot} \text{ km s}^{-1}$ estimated for a supernova remnant. These estimates are fairly uncertain. The momentum in the [Fe II] knots is 4–5 orders of magnitude smaller again.

Whether molecular shocks are excited through hydrodynamic jump-shocks or magnetically mediated continuous-shocks has been much debated^{29,30}. Some form of bow-shock model³¹ is necessary to account for the constancy of H_2 line ratios with position³² and the $\sim 150 \text{ km s}^{-1}$ wide profiles²⁸. Discussion^{33,34} has focused on the brighter emission regions where bow shocks, if present, must overlap. Spatially resolved profile and line ratio measurements in the outer bows should resolve this debate.

It would be dangerous to infer from this single example that all HH objects associated with young outflows require explosive ejection. There is strong evidence that some HH objects form when jets from young stars impinge on ambient cloud material. Rather, this discovery highlights the need to search other star-forming regions to establish the statistics on such events. □

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Superconductivity above 130 K in the Hg–Ba–Ca–Cu–O system

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THE recent discovery¹ of superconductivity below a transition temperature (T_c) of 94 K in $\text{HgBa}_2\text{CuO}_{4+\delta}$ has extended the repertoire of high- T_c superconductors containing copper oxide planes embedded in suitably structured (layered) materials. Previous experience with similar compounds containing bismuth and thallium instead of mercury suggested that even higher transition temperatures might be achieved in mercury-based compounds with more than one CuO_2 layer per unit cell. Here we provide support for this conjecture, with the discovery of superconductivity above 130 K in a material containing $\text{HgBa}_2\text{Ca}_2\text{Cu}_3\text{O}_{1+\delta}$ (with three CuO_2 layers per unit cell), $\text{HgBa}_2\text{CaCu}_2\text{O}_{6+\delta}$ (with two CuO_2 layers) and an ordered superstructure comprising a defined sequence of the unit cells of these phases. Both magnetic and resistivity measurements confirm a maximum transition temperature of ~ 133 K, distinctly higher than the previous established record value of 125–127 K observed in $\text{Ti}_2\text{Ba}_2\text{Ca}_2\text{Cu}_3\text{O}_{10}$ (refs 2, 3).

The structural similarity of $\text{HgBa}_2\text{CuO}_{4+\delta}$ (Hg-120; ref. 1) to a member of the thallium-containing family of copper oxides, $\text{TlBa}_2\text{CuO}_5$ (Tl-1201), suggests the existence of compounds with the general composition $\text{HgBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+2+\delta}$. The transition temperatures of the thallium-containing analogues, $\text{TlBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+3}$, range from <10 K ($n=1$, ref. 4) to ~ 110 K ($n=3$, ref. 5). In this sense, transition temperatures exceeding 100 K may be expected also in the Hg–Ba–Ca–Cu–O

(HBCCO) system. Although the successful synthesis of $\text{HgBa}_2\text{RCu}_2\text{O}_{6+x}$ (Hg-1212) with R being (Eu, Ca) has been reported, no superconductivity was found in that system⁶.

We prepared the samples following the procedure described in ref. 1 for Hg-1201. A precursor material with the nominal composition $\text{Ba}_2\text{CaCu}_2\text{O}_5$ was obtained from a well ground mixture of the respective metal nitrates, sintered at 900 °C in O_2 . After regrinding and mixing with powdered HgO , the pressed pellets were sealed in evacuated quartz tubes. These tubes were placed horizontally in tight steel containers and held at 800 °C for 5 hours. On opening the containers, we found that the quartz tubes were broken. It was not possible to reconstruct at which stage of the heating, cooling or opening procedure this happened. Some of the pellets were finally annealed for 5 hours at 300 °C in flowing oxygen. During the preparation and the characterization of the samples, all possible measures were taken to avoid any contamination with toxic mercury or mercury-containing compounds.

After annealing, the resulting black material was characterized by X-ray diffraction using the Guinier technique, by energy-dispersive X-ray spectrometry (EDS), and by selected-area electron-diffraction techniques (SAED) and high-resolution transmission electron microscopy (HRTEM). The EDX analysis showed that the samples are composites of isolated grains of BaCuO_2 (~30%), CuO (~30%), an unidentified oxide containing Hg, Ca and Cu (~15%), an oxide with Ca and Cu (~5%), and ~5% impurities with unspecified composition. About 15% of the total sample volume consisted of plate-like grains containing Hg, Ba, Ca and Cu. Some of these were investigated in detail by SAED and HRTEM techniques on a Phillips CM 30-ST transmission electron microscope. Both techniques showed clearly that these identified grains consist mostly of pure $\text{HgBa}_2\text{Ca}_2\text{Cu}_3\text{O}_{8+x}$ (Hg-1223), disordered mixtures of Hg-1223 and Hg-1212, and a periodic stacking sequence of the latter unit cells. We found no grains or intergrowths associated with the Hg-1201 structure. As the volume fraction of the phases of interest is fairly small, we could not measure the lattice parameters precisely with the X-ray Guinier technique. Nevertheless, from the SAED patterns, we deduce the lattice constants $c = 12.7(2)$ Å and $c = 16.1(3)$ Å for the tetragonal Hg-1212 and Hg-1223 units, respectively, and $a = 3.93(7)$ Å, valid for both types

of compounds. The results for Hg-1212 are in good agreement with the values obtained in ref. 6. Figure 1 is a representative HRTEM image showing, as an example, a stacking containing both Hg-1212 and Hg-1223 layers. The stacking sequence 1223/1223/1212/1212/1223/1212 with a supercell c -axis $c \approx 86.4$ Å extends beyond 2,000 Å, thus qualifying this superstructure as a proper phase. HRTEM images as well as SAED patterns gave no evidence for the presence of HgO -double layers.

We measured the magnetic susceptibility of the specimens using a SQUID-magnetometer (Quantum Design). Figure 2 shows the result obtained for an oxygen-annealed sample. In an external field $H = 27$ Oe, the zero-field cooling susceptibility (ZFC) amounts to ~100% of $1/4\pi$ at temperature $T = 6$ K, indicating complete magnetic screening. For this estimate, we

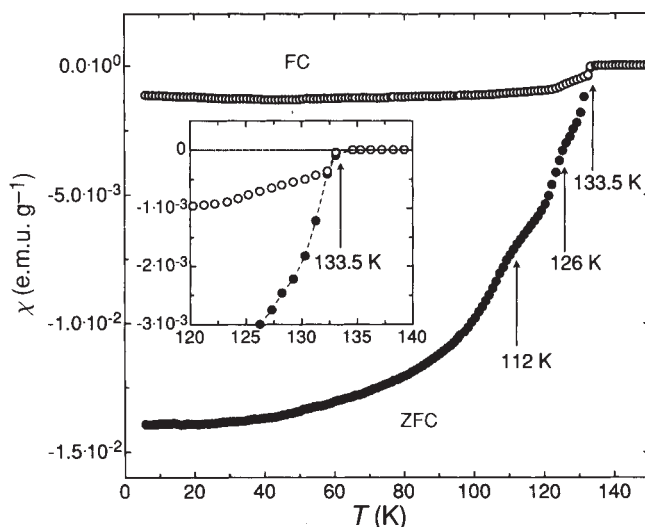


FIG. 2 Zero-field cooling (ZFC) and field cooling (FC) susceptibilities $\chi(T)$ of one of the investigated oxygen-annealed HBCCO samples, measured in $H = 27$ Oe. The ZFC curve indicates the presence of several different superconducting phases.

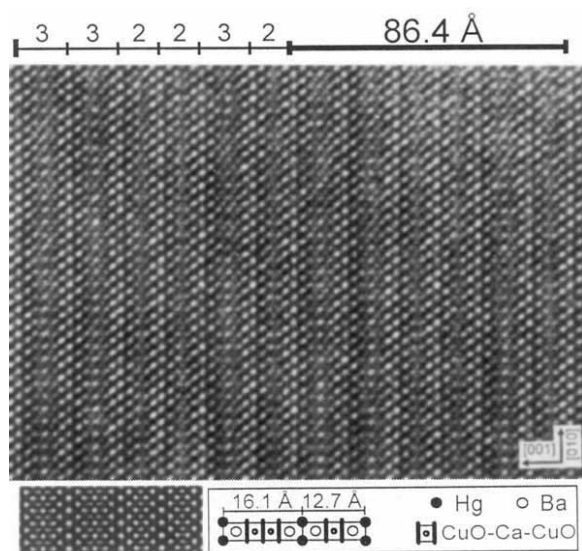


FIG. 1 HRTEM image of a grain in [100] orientation, containing layers of Hg-1212 and Hg-1223. Here, they are stacked in a periodic sequence forming a supercell with $c \approx 86.4$ Å (see text). A contrast simulation ($c_s = 1.1$ mm, $E = 300$ keV, defocus -870 Å, specimen thickness 23 Å) is inserted. The stacking sequence in terms of the number of Cu-O planes and an enlarged schematic drawing of the involved unit cells are included.

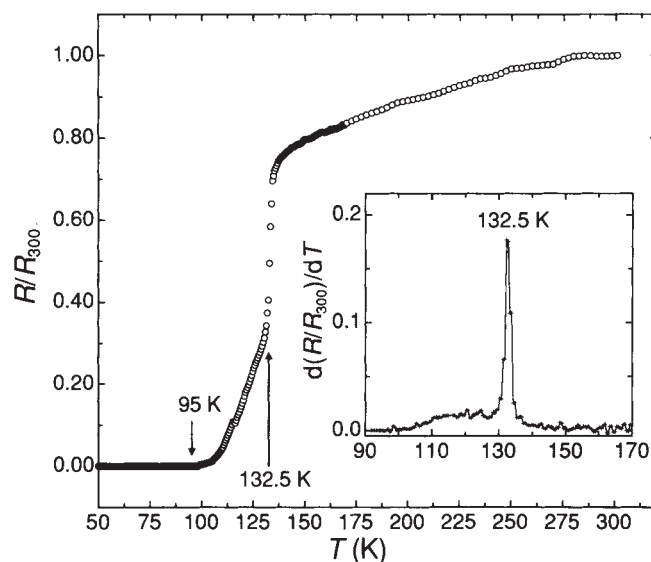


FIG. 3 Resistivity $R(T)$ of an annealed HBCCO specimen, normalized with respect to the resistance value $R(300) \approx 0.10$ Ω. The inset displays the temperature derivative dR/dT to show the maximum resistivity drop at $T \approx 132.5$ K. Zero resistance is attained at $T = 95$ K.

assumed an average density $\rho \approx 6 \text{ g cm}^{-3}$. The field-cooling (FC) susceptibility reaches $\sim 10\%$ of the maximum possible value. This value represents a lower-bound value for the true superconducting volume fraction in the sample, indicating the bulk nature of superconductivity. The onset temperature of diamagnetism is $T_c \approx 133.5 \text{ K}$, seen both in FC and ZFC experiments (see Fig. 2, inset). The FC susceptibility reaches $\sim 60\%$ of its full low-temperature value at 125 K , strongly indicating that the phase with $T_c \approx 133.5 \text{ K}$ dominates all other superconducting phases. In the ZFC curves, additional features are seen at 126 K and 112 K , which we ascribe to different superconducting phases with lower transition temperatures.

The resistivity R as a function of temperature T of an annealed sample is shown in Fig. 3. At $T \approx 132.5 \text{ K}$, $R(T)$ drops sharply with a maximum in the differential dR/dT , and reaches zero at $T = 95 \text{ K}$ within the resolution of the four-probe a.c.-resistance bridge used. This temperature is still considerably higher than the zero-resistance temperature $T \approx 35 \text{ K}$, reported for Hg-1201 (ref. 1). The final oxygen treatment was very effective in increasing the critical temperature; the as-sintered samples showed a maximum T_c of only $\sim 117 \text{ K}$.

At present we cannot relate the different superconducting phases to crystallographic phases. There is no unambiguous proof that the occurrence of superconductivity in our samples stems from the $\text{HgBa}_2\text{Ca}_{n-1}\text{Cu}_n\text{O}_{2n+2+\delta}$ phases. In analogy with the thallium- and bismuth-based copper oxides⁵, however, we suggest that in the HBCCO system T_c also increases with the number of Cu-O planes per unit cell, and conclude that Hg-1223 is responsible for superconductivity at $\sim 133 \text{ K}$. This would be consistent with the large relative superconducting volume fraction at 125 K , in view of the dominance of Hg-1223 observed in the grains investigated microscopically. $\square$

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Large second-order optical polarizabilities in mixed-valency metal complexes

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THE potential development of optoelectronic devices based on the nonlinear polarization of molecular materials has aroused much recent interest^{1,2}. The search for large second-order electric susceptibilities (that is, proportional to the square of an applied electric field) has concentrated on acentric organic or organometallic chromophores with an organic π -electron system coupling electron donor and acceptor groups^{3–6}. It is conceivable that mixed-valence compounds characterized by an intervalence charge-transfer (IVCT) transition⁷, in which the donor and acceptor centres are both metal atoms, might also have the potential to provide a large second-order response⁸, but this possibility has not been widely explored. Here we report the first hyperpolarizability, β , of a bimetallic complex ion, $[(\text{CN})_5\text{Ru}-\mu\text{-CN}-\text{Ru}(\text{NH}_3)_5]^-$ (I in Fig. 1), and a novel organometallic analogue, $[(\eta^5\text{-C}_5\text{H}_5)\text{Ru}(\text{PPh}_3)_2-\mu\text{-CN}-\text{Ru}(\text{NH}_3)_5]^{3+}$ (II). Measurements of β (which is related to the bulk second-order response) in solution at a wavelength of $1,064 \text{ nm}$ using the newly developed hyper-Rayleigh scattering technique^{9,10} give values greater than 10^{-27} e.s.u. , which are among the largest reported for solution species. The ease with which the energy of the IVCT transition can be modified suggests that there may be considerable potential for this class of chromophore in nonlinear optical devices.

The polarization induced by an intense laser beam includes components that are nonlinear in the optical field¹¹. In molecular materials, the susceptibility tensor $\chi^{(2)}$, which determines the second-order response, can be related to underlying molecular hyperpolarizability tensors, β . There are important applications for materials with large values of $\chi^{(2)}$, such as the frequency-doubling of red III–V semiconductor lasers to provide compact blue sources for high-density optical storage¹², and the electro-optical modulation and switching of telecommunications signals¹³; both applications require waveguide device structures.

In centrosymmetric molecules β is zero, but even for non-centrosymmetric molecules with large hyperpolarizability the bulk susceptibility $\chi^{(2)}$ vanishes unless the distribution of molecular orientations is also acentric¹¹. A large value of β is, therefore, the first priority in an efficient molecular material, whether it be organic, organometallic or inorganic, but to maximize $\chi^{(2)}$ it is also essential to establish the appropriate molecular alignment. To achieve this in crystalline materials, whilst also defining a waveguide structure of high optical quality,

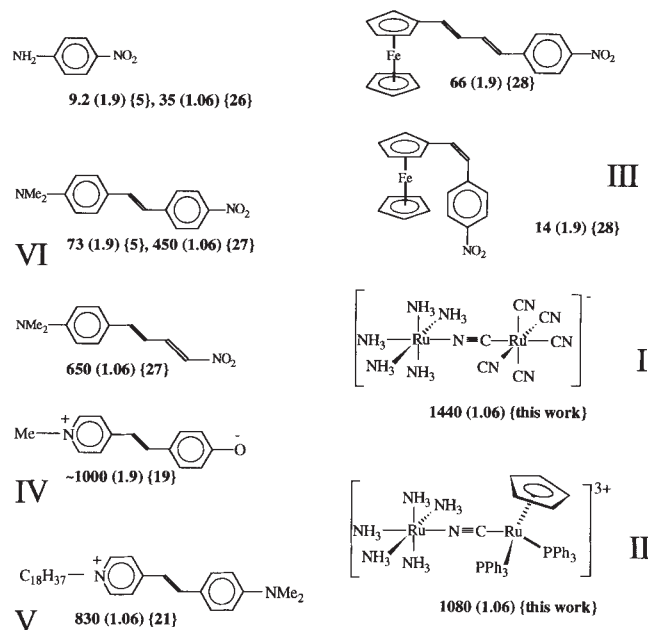


FIG. 1 The first number near each molecule is the magnitude of β in units of 10^{-30} e.s.u. The wavelength of measurement in μm is shown in parentheses. References are enclosed in brackets. The complex anion I was purified by first cation and then anion exchange chromatography, and recrystallized from aqueous methanol. The product is the lithium trihydrate salt, $[(\text{CN})_5\text{Ru}-\mu\text{-CN}-\text{Ru}(\text{NH}_3)_5]^- \cdot \text{Li} \cdot 3\text{H}_2\text{O}$. Analysis for $\text{C}_6\text{H}_{21}\text{LiN}_{11}\text{O}_3\text{Ru}_2$ ($M_r = 504.1$) gives the following results. Found/(Calc. %): C = 14.6(14.3), H = 4.1(4.2), N = 30.3(30.6). The *tris*-trifluoromethanesulphonate salt of II was prepared by reacting $[(\eta^5\text{-C}_5\text{H}_5)\text{Ru}(\text{PPh}_3)_2\text{CN}]$ with $[\text{Ru}(\text{NH}_3)_5(\text{OSO}_2\text{CF}_3)](\text{CF}_3\text{SO}_3)_2$ in acetone, followed by recrystallization from a mixture of dichloromethane and diethylether, to give $[(\eta^5\text{-C}_5\text{H}_5)\text{Ru}(\text{PPh}_3)_2-\mu\text{-CN}-\text{Ru}(\text{NH}_3)_5](\text{CF}_3\text{SO}_3)_3$. Analysis for $\text{C}_{45}\text{H}_{50}\text{F}_9\text{N}_6\text{O}_9\text{P}_2\text{Ru}_2\text{S}_3$ ($M_r = 1350.16$) gives found/(Calc. %): C = 40.0(40.0), H = 3.7(3.7), N = 6.0(6.2).

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is possible but not easy. An attractive alternative is to orient the nonlinear chromophore, contained in a polymeric host, by means of a strong d.c. electric field applied above the glass transition temperature. The induced polarity can be frozen in by cooling or polymer cross-linking, and is more durable if the nonlinear entity is chemically bonded to the polymer backbone¹⁴. Ideally the susceptibility is proportional to the product of β and the dipole moment, μ , that determines the orientational torque. The fabrication of waveguides in thin polymeric films is relatively easy, and a 40-GHz modulator has been demonstrated¹⁵.

The dependence of the hyperpolarizability, β , on organic structure has been extensively explored^{4-6,16}; some examples are shown in Fig. 1, along with the mixed-valence complexes studied here. The limited flexibility of organic donors and acceptors, and the observation¹⁷ of considerable second-harmonic generation (SHG) in compound **III**, has drawn attention to organometallics, but their β values prove to be unexceptional^{18,19}. The merocyanine dye, **IV**, has the largest solution value of β . Electric-field-induced SHG²⁰ measurements, at 1.89 μm , give $\beta \approx 10^{-27}$ e.s.u. ($\beta(\text{SI}) = 4.189 \times 10^{-10} \beta$ (e.s.u.)) but this result is based on an estimated dipole moment. A monolayer Langmuir-Blodgett (LB) film derived from **IV** is reported to have $\beta = 4,000 \pm 130 \times 10^{-30}$ e.s.u., but the layer is not stable and the value depends on assumptions about its structure²¹. The related hemicyanine, **V**, which is ordered in LB multilayers, gives $\beta = 830 \pm 350 \times 10^{-30}$ e.s.u. at 1.06 μm (ref. 22).

The use of mixed-valence compounds (MVCs) as nonlinear optical (NLO) chromophores was suggested by Lehn⁸. Here we report the properties of $\text{Ru}^{\text{II}}/\text{Ru}^{\text{III}}$ species linked by a cyanide ion. In the complex ions **I** and **II** (see Fig. 1), both ruthenium ions are inert to ligand substitution. Charge asymmetry is established by stabilizing the $\text{Ru}^{\text{II}}(d_{\pi}^6)$ with π -acid ligands and $\text{Ru}^{\text{III}}(d_{\pi}^5)$ with aminines. These are class II MVCs⁷, retaining charge localization, but with enough interaction between the centres to permit intense IVCT transitions; we have synthesized compounds in which other metals and ligands have been used to tune the energy of the IVCT. The anion **I** was synthesized by the method of Vogler *et al.*²³, purified by anion exchange

chromatography, and characterized as the hydrated lithium salt. Ion **II** was synthesized by direct coupling.

Electric-field-induced SHG cannot be applied to conducting solutions, but the recent development of hyper-Rayleigh scattering (HRS), has made it possible to measure β for ions as well as for neutral molecules. In our HRS measurements^{9,10}, an injection-seeded, Q-switched Nd-YAG laser pulse at 1,064 nm is focused into a 4-cm cell. Within a volume whose length is of the order of the wavelength, the instantaneous average of the molecular orientation has a non-zero polar component. There is, therefore, a finite second-order susceptibility, enabling second-harmonic scattering at 532 nm, with an intensity at the detector, $I_{2\omega}$, given by

$$I_{2\omega}/(I_{\omega}^0)^2 = G(N_s\beta_s^2 + N_c\beta_c^2) e^{-N_c(2\sigma_{\omega}l_1 + \sigma_{2\omega}l_2)} \quad (1)$$

where I_{ω}^0 is the incident intensity, G is a collection efficiency, N_s and N_c are solvent and solute number densities, σ_{ω} and $\sigma_{2\omega}$ are absorption cross-sections, l_1 and l_2 are effective absorption pathlengths for the fundamental and second harmonic respectively, and β_s and β_c are the hyperpolarizabilities. Calibration is relative to the pure solvent. When the absorption cross-sections are non-zero, as in this case, the data are fitted to equation (1). This is shown in Fig. 2; the maximum is due to increasing absorption with concentration. Using experimental absorption cross-sections and $l_1 = 2$ cm, l_2 is found to be of the order of the transverse cell dimensions, so that the basis of the analysis is self-consistent. For **I** we find $\beta = 1,440 \pm 200 \times 10^{-30}$ e.s.u. in aqueous solution, and for **II** $\beta = 1,080 \pm 200 \times 10^{-30}$ e.s.u. in nitromethane solutions.

The optical polarization can be described by a superposition of a set of excited electronic states; they may be divided into those created by excitations centred on a single metal ion, and those created by IVCT. The single-centre contribution to β in **I** should be small because both ruthenium ions are in nearly centrosymmetric sites; monomeric analogues typically have $\beta < 10 \times 10^{-30}$ e.s.u. (ref. 18). In single crystals of the lithium salt, the IVCT band, whose maximum is near 14,000 cm^{-1} , is polarized parallel to the intermetallic axis, the initial and final states being derived from the configurations $d_{A\pi}^6 d_{B\pi}^5$ and $d_{A\pi}^5 d_{B\pi}^6$. We therefore attribute the large values of β to the IVCT excited states, together with near-resonant enhancement caused by the proximity of the optical and IVCT transition frequencies.

The energy of the IVCT band is sensitive both to the solvent (Fig. 3) and to solid-state interactions; the nitrate and dihydrogenphosphate salts of **II** are purple, whereas the trifluoromethanesulphonate salt is blue. This sensitivity could

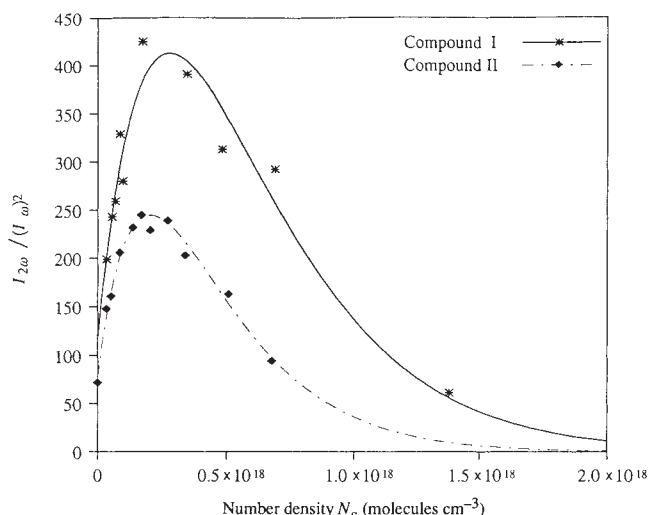


FIG. 2 The hyper-Rayleigh scattering efficiency, in arbitrary units, as a function of sample concentration. The curves are refined fits (with $R > 0.98$) to equation (1). The light scattered from a solution irradiated with 100-mJ, 10-ns pulses at 1,064 nm is passed through a narrow-band interference filter centred at 532 nm and an infrared blocking filter. The photomultiplier signal and a reference photodiode signal are averaged by gated integrators. Each data point corresponds to a least-squares fit of the quadratic dependence of $I_{2\omega}$ on I_{ω}^0 over a wide dynamic range.

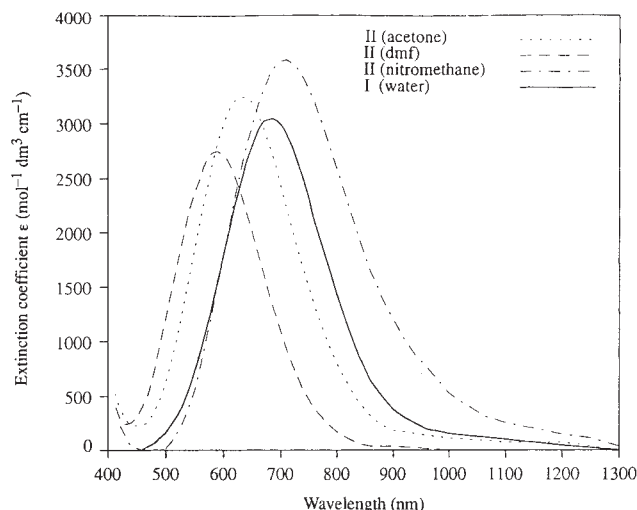


FIG. 3 Electronic absorption spectra of **I** and **II**, showing the dependence of the IVCT band position on the solvent.

assist the reduction of absorption losses. If a molecular chromophore is to be oriented in a polymeric medium, in order to support the poling field it must be electrically neutral. In I this could be achieved by replacing a terminal cyanide ion by a neutral ligand. The metal centres, which we estimate are separated by 520 pm, carry formal charges of +2.5 and -2.5, so that $\mu \approx 52$ Debye, whereas the typical organic chromophore *N,N*-dimethylamino-4-nitrostilbene (DANS; VI in Fig. 1) has $\mu = 6.6$ Debye⁵. Using the method of Hush^{24,25}, we calculate charge delocalization across the bridge to be less than 2%, so μ should be close to our estimate. Such a large polarity could prove to be helpful in establishing a high degree of orientational order in poled films. □

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Experimental evidence for the formation of fullerenes by collisional heating of carbon rings in the gas phase

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THE discovery^{1–6} of the spherical carbon cage compound buckminsterfullerene (C_{60}) and the recent development of methods to produce it in bulk⁷ have led to an explosion in research in the physical and chemical properties of this unique species^{8,9}. Nevertheless, the question of the formation mechanism of C_{60} (or of the other fullerenes) is still far from settled. We have shown elsewhere that carbon clusters in the gas phase develop from linear chains to planar ring systems to fullerenes as their size increases. One can easily envisage the transformation from chains to rings, but how the three-dimensional near-spherical fullerenes evolve from large planar rings is not obvious. Here we show that 'heating' these large ring systems above their 'melting' point leads to 100% fullerene formation accompanied by the evaporation of a small carbon fragment (C_1 or C_3 for odd systems and C_2 for even systems). We propose a mechanism, based on these data, for efficient C_{60} production in carbon arcs.

Early suggestions for the formation mechanism of fullerenes^{1–4} focused on curling up of graphitic sheets that grow by the addition of C_2 units. More recently, Heath¹⁴ has proposed the 'fullerene road' scenario, in which fullerenes appear (according to an unspecified mechanism) around C_{40} and grow by sequential addition of C_2 units, eventually stopping at C_{60} where further addition becomes difficult. Smalley¹⁵, in contrast has favoured the 'pentagon road', which is really a variant of the earlier curling-graphitic-sheet model, except that pentagons appear, because of annealing, in just the right positions so that closure to form C_{60} occurs naturally and defects, overshooting and so forth are minimized. 'Ring-stacking' is another recent model¹⁶, where an appropriate number of rings of different sizes are stacked, eventually forming C_{60} . Rubin *et al.*¹⁷ and

McElvany *et al.*¹⁸ have shown by mass spectrometry that (presumably fullerene) C_{60} and C_{70} can be formed in the gas phase from C_{18} , C_{24} and C_{30} cyclic all-carbon precursors by ion-molecule reactions. These studies suggest a cationic pathway in the formation process from condensation of large ring systems. Curl¹⁹ has recently summarized and analysed some of these models.

We have recently developed a gas-phase ion chromatography technique²⁰ and applied it to carbon cluster cations^{10,11} and anions^{11–13}. A pulse of mass-selected cluster ions is injected into a high-pressure drift cell filled with 2–5 torr of helium. The ionic mobilities of different isomeric structures depend on their different collision cross-sections with He, and the isomers are therefore separated while drifting through the cell, under the influence of a weak electric field. The absolute value of the ionic mobility for a given cluster together with computer simulations often allows unambiguous determination of the cluster

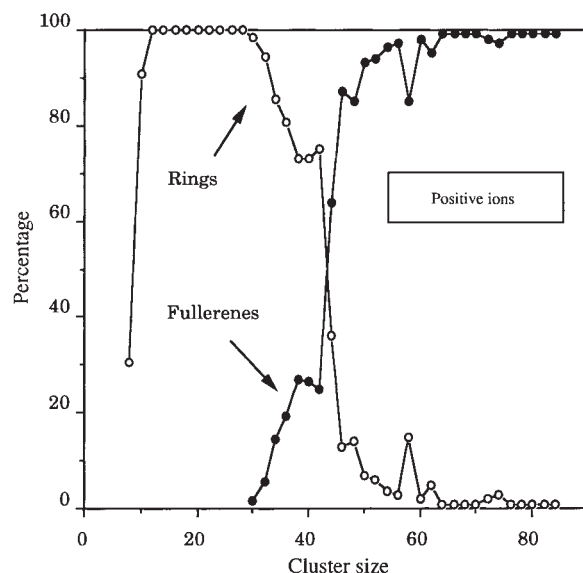


FIG. 1 Plots of percentage of planar rings and fullerenes against carbon cluster size. Experimental details are given in the text and in refs 10–13. Only the even cluster ions are shown for clarity. The odd cluster positive ions yield curves with a similar shape, but the planar rings decrease less quickly as size increases, and make up about 40% of the total near C_{60} .

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structure^{12,13}. The method also allows us to determine the carbon cluster isomer abundances as a function of cluster size. We have found that small clusters are linear; near C_{10} , planar monocyclic rings appear, which gradually give way to planar bicyclic rings between C_{20} and C_{30} (refs 12, 13); finally planar tricyclic rings evolve in the C_{30} s and dominate the ring systems thereafter¹³. Fullerenes first appear at C_{30} for the cations and dominate by C_{50} . For negative ions the planar multicyclic rings dominate to well above C_{60} . In Fig. 1 these trends are quantified by plotting the fraction of planar rings and fullerenes against cluster size for clusters made in our laser vaporization source.

Here we focus on the transition region between C_{30}^+ and C_{40}^+ , as fullerenes first appear in this range. The progression linear $\rightarrow$ monocyclic rings $\rightarrow$ bicyclic rings $\rightarrow$ tricyclic rings can readily be understood: at a certain minimum size, energized linear molecules can close and form a ring and then these rings can react with each other to form bicyclic and tricyclic rings. But where do the fullerenes come from? We find no evidence^{11,12} for fullerenes or 'graphitic' or 'cup'-like fullerene precursors in our chromatography data for ions below C_{30}^+ , yet we see large abundances of fullerenes for larger clusters¹³. There seems to be no simple way the families of planar cyclic rings can suddenly switch to fullerenes simply by adding new members.

It occurred to us that our source might not be providing long enough periods of high temperatures for structural 'annealing'

to occur. We therefore tried a variant of an experiment successfully applied on silicon clusters²¹. To generate the data shown in Fig. 1, a narrow pulse of mass-selected ions was injected into the chromatography cell at very low energy (2 to 20 eV), and the ions were rapidly thermalized by the 5 torr of He present. In our 'annealing' studies, in contrast, the ions were intentionally injected at fairly high voltages, creating a transient internal energy increase that can allow isomerization, or even collision-induced dissociation. In both cases, the arrival time distribution (ATD) at the detector gives the isomer distribution when the quadrupole (following the chromatography cell) is tuned to the appropriate mass. The results for C_{39}^+ and C_{40}^+ are shown in Fig. 2a and b respectively. At low injection energies (Fig. 2a, panel A), the C_{39}^+ ATD is dominated by planar bicyclic and tricyclic ring systems, but at high injection energies those C_{39}^+ ions that survive (those not dissociated by the internal energy surge) are converted essentially 100% to the monocyclic ring isomer (Fig. 2a, panel B). The three-dimensional ring and fullerene are essentially unaffected. Clearly the monocyclic ring is the most stable planar ring structure. This result does not give any information on fullerene formation, but by scanning the quadrupole mass filter following the chromatography cell, we noticed that a substantial fraction of the C_{39}^+ ions injected at 150 eV had been collisionally dissociated.

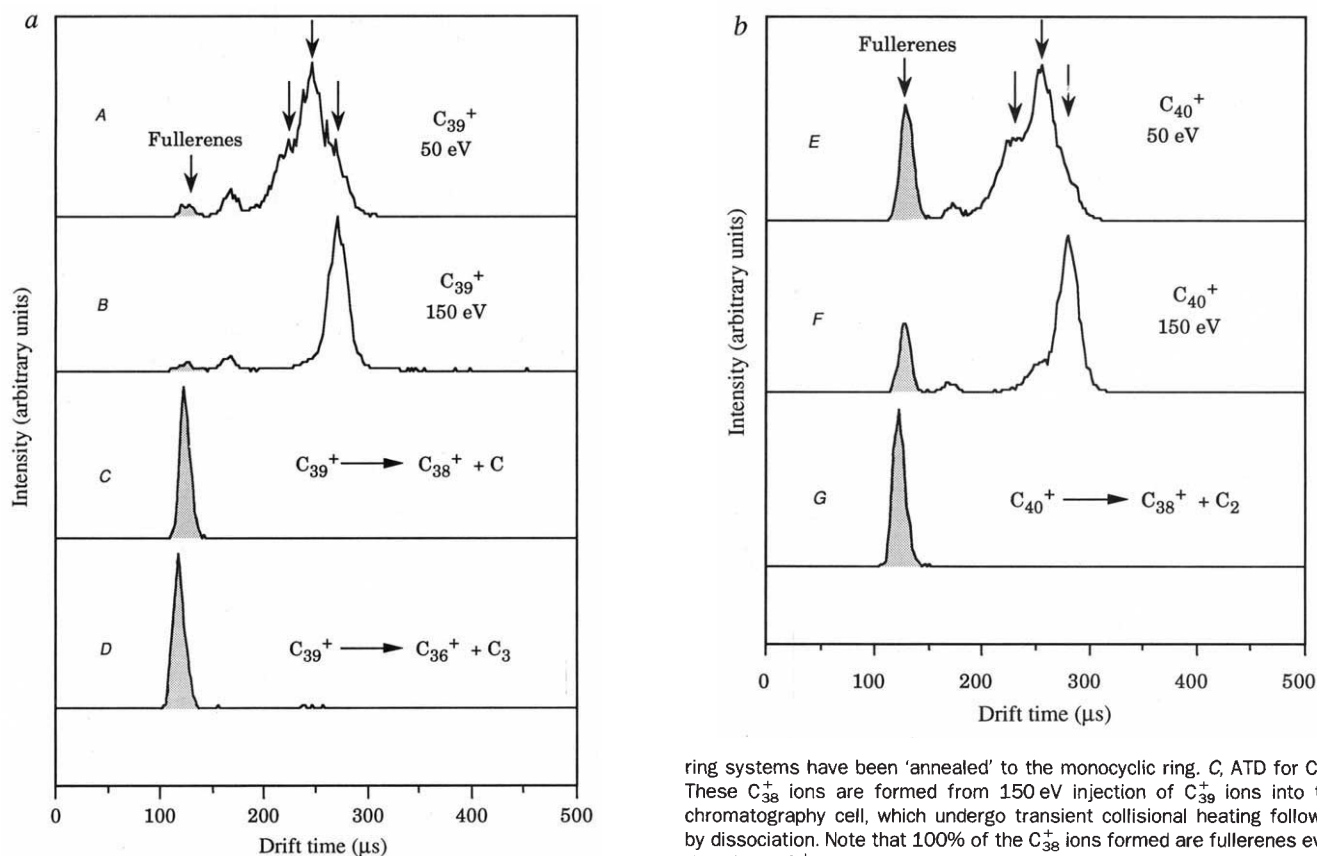
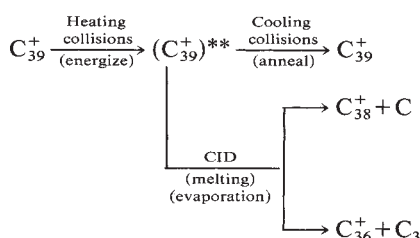
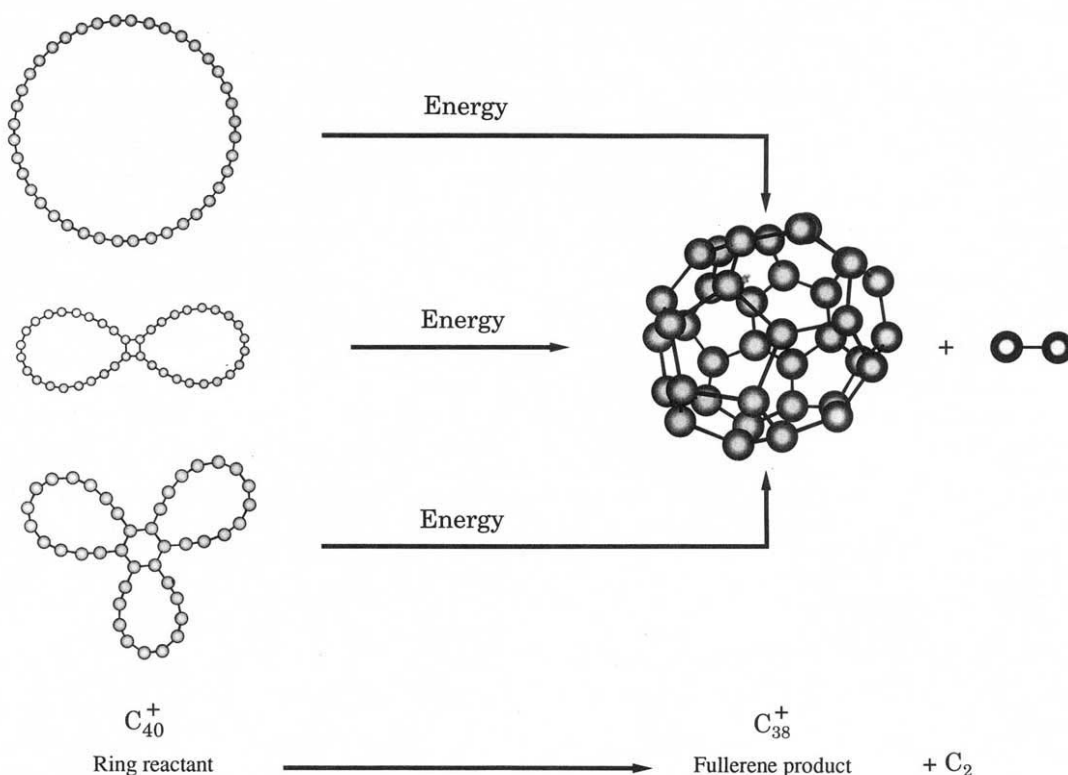


FIG. 2 Arrival time distributions (ATD; ion chromatograms) obtained under different conditions (see text and refs 10–13). a, Results for C_{39}^+ . A, C_{39}^+ injected at 50 eV into the cell. This distribution is essentially identical to those injected at 2–3 eV. The three arrows over the large composite peak indicate the presence of monocyclic planar rings (longest time) bicyclic planar rings (middle) and tricyclic planar rings (shortest time of this peak). The small shaded peak near 120 μs is the C_{39}^+ fullerene. The remaining peak near 160 μs is a three-dimensional ring structure. D, ATD of C_{36}^+ ions formed from collision-induced dissociation of C_{39}^+ . These C_{36}^+ ions are essentially 100% fullerene. b, Results for C_{40}^+ . E, ATD of C_{40}^+ injected into the chromatography cell at 50 eV is given. This ATD is very similar to one obtained by injection at much lower voltages. The arrows over the composite peak at longest times indicate the presence of monocyclic, bicyclic and tricyclic ring systems. The shaded peak at shortest times (near 120 μs) is the C_{40}^+ fullerene peak. F, the ATD for surviving C_{40}^+ ions injected at 150 eV. Annealing of the planar ring systems leads predominantly to the monocyclic ring isomer. G, ATD for C_{38}^+ ions. These are obtained by injection of C_{40}^+ ions into the chromatography cell, followed by loss of C_2 . Clearly, the resultant C_{38}^+ ions are 100% fullerene.

ring systems have been 'annealed' to the monocyclic ring. C, ATD for C_{38}^+ . These C_{38}^+ ions are formed from 150 eV injection of C_{39}^+ ions into the chromatography cell, which undergo transient collisional heating followed by dissociation. Note that 100% of the C_{38}^+ ions formed are fullerenes even though the C_{39}^+ parent ions were almost exclusively planar rings. D, ATD of C_{36}^+ ions formed from collision-induced dissociation of C_{39}^+ . These C_{36}^+ ions are essentially 100% fullerene. b, Results for C_{40}^+ . E, ATD of C_{40}^+ injected into the chromatography cell at 50 eV is given. This ATD is very similar to one obtained by injection at much lower voltages. The arrows over the composite peak at longest times indicate the presence of monocyclic, bicyclic and tricyclic ring systems. The shaded peak at shortest times (near 120 μs) is the C_{40}^+ fullerene peak. F, the ATD for surviving C_{40}^+ ions injected at 150 eV. Annealing of the planar ring systems leads predominantly to the monocyclic ring isomer. G, ATD for C_{38}^+ ions. These are obtained by injection of C_{40}^+ ions into the chromatography cell, followed by loss of C_2 . Clearly, the resultant C_{38}^+ ions are 100% fullerene.

FIG. 3 Planar monocyclic, bicyclic and tricyclic C_{40}^+ ions form C_{38}^+ fullerene plus C_2 when collisionally heated above the isomerization barrier for fullerene formation (see Fig. 2b). All structures shown are minima on the potential energy surface, according to semi-empirical PM3 electronic structure calculations. The bicyclic and tricyclic rings have lower isomerization barriers to fullerene formation than the monocyclic ring.



The isomer chromatograms of the C_{36}^+ and C_{38}^+ fragments are given in Fig. 2a (panels C and D). The results are unambiguous: in both instances, the products are essentially 100% fullerene. This process is represented pictorially in Fig. 3, where C_{38}^+ fullerene is created by heating C_{40}^+ ring systems.

It seems that when the C_{39}^+ or C_{40}^+ planar multicyclic ring ions gain insufficient energy to rearrange to a fullerene, they rearrange by breaking one or two bonds and form the more stable monocyclic ring structure. This is not common in the ion source because when these multi-ring species are first formed (presumably through ring-ring collisions), rapid cooling quenches the cluster products and results in a metastable isomer distribution. By injecting this distribution into the chromatography cell at high voltage, however, we induce a strong transient heating pulse. Once the C_{39}^+ or C_{40}^+ planar ring ions are collisionally 'heated' above the threshold for isomerization to a fullerene, rapid bond breaking and rearrangement occurs and the cluster begins to relax to its most stable form. At this point the internal energy in the cluster can be enormous. For example, the calculations of ref. 22 indicate that conversion of bicyclic planar C_{36} to a C_{36} fullerene liberates ~ 9 eV, and conversion of a tricyclic planar C_{60} ring system to C_{60} liberates ~ 45 eV. If one adds to this the internal energy obtained in the injection pulse²¹ (5–20 eV), vibrational temperatures near 4,000 K are indicated; this can be compared with the carbon melting temperature of 3,740 K. The superheated C_{39}^+ 'fullerene' ion cools itself by 'evaporating' either a C atom or a C_3 molecule, leaving behind a C_{38}^+ or C_{36}^+ fullerene, whereas C_{40}^+ loses C_2 to form C_{38}^+ fullerene.

This fullerene formation process starts gradually with C_{33}^+ , where a small amount of C_{30}^+ fullerene is made by loss of C_3 . Theory^{22,23} predicts that fullerenes become more stable than planar rings near C_{30} , in support of our observations. The process rapidly accelerates with increasing size, as is evident from Figs 1 and 2. This result is consistent with the fact that for carbon cations larger than C_{40}^+ , the fullerene isomer dominates (Fig. 1). We conclude from these data that the barrier for transforming planar ring systems to fullerenes decreases with size, a result consistent with the rapid increase in stability of fullerenes relative to rings in this size range²².

A comment about the C_{60} formation in carbon arc reactors is in order. Experiments^{24–27} using C_{12}/C_{13} graphite unambiguously showed that C_{60} is synthesized starting from carbon atoms. Efficient synthesis of C_{60} occurs only for a narrow range of experimental parameters (pressure, rod feed rate and so on) so the kinetics must be fairly specific. Our chromatography experiments seem to have ruled out a graphitic-like growth mechanism, such as the 'pentagon road', as we have shown^{10–13} that carbon initially forms linear chains, followed by planar ring systems as it grows. We have shown here that collisional heating of large planar ring systems induces isomerization to fullerenes. It seems possible (or even probable) that in arc reactors the atomic carbon 'soup' diffuses out of the arc, and initially forms chains and then rings. The carbon density immediately surrounding the arc may be high enough that these rings rapidly coalesce to sizes larger than C_{60} and spontaneously rearrange to fullerenes because of the low rearrangement barrier for large systems and the hot environment. Evaporation of small particles from the newly formed fullerene would preferentially yield C_{60} , the most stable species in the immediate size range. It is reasonable that the growth of planar rings to the size that will eventually form C_{60} would be maximized under specific experimental conditions in the arc reactor, otherwise smaller (or larger) fullerenes would be formed, as shown in Fig. 1. The abundance of C_{60} could be further enhanced by growth of smaller fullerenes, formed from isomerization of smaller ring systems, via the

'fullerene road', although adequate supplies of small carbon fragments and several successive steps are required for this to occur.

The data presented here unlock the mystery of how fullerenes appear near C_{30} in laser desorption experiments when no graphitic building blocks are present at smaller sizes. The ring $\rightarrow$ fullerene isomerization mechanism might also help explain the rather surprising propensity for C_{60} formation in carbon arc reactors. □

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Uranium–thorium disequilibria and partitioning on melting of garnet peridotite

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THE abundances of isotopes in the ^{238}U decay series can be used as both tracers and chronometers of magmatic processes. In the subsolidus asthenosphere, the activity of each daughter isotope (defined as the product of its concentration and decay constant, and denoted by parentheses) is assumed to be equal to that of its parent. By contrast, ($^{230}\text{Th}/^{238}\text{U}$) is greater than unity in most recent mid-ocean-ridge and ocean-island basalts¹, implying that thorium is more incompatible (that is, it is partitioned into the melt phase more strongly) than uranium. Melting of spinel peridotite cannot produce the (^{230}Th) excesses, because measured partition coefficients for pyroxenes and olivine demonstrate that uranium is more incompatible than thorium for this rock². Here I report garnet–melt partitioning data which show that for this mineral–melt pair thorium does behave more incompatibly than uranium, thus supporting the suggestion that mid-ocean-ridge basalts (MORB) are produced by melting initiated at depths where garnet is stable^{3–6}. Using these data, I show that the observed ($^{230}\text{Th}/^{238}\text{U}$) ratios of MORB and most ocean-island basalts can be explained by slow, near-fractional melting initiated in the garnet stability field.

TABLE 1 Typical phase compositions for mixtures A, B, C and D

Oxide	Mixture A		Mixture B		Mixture C		Mixture D	
	gt	L	gt	L	gt	L	gt	L
SiO ₂	37.1	42.7	37.3	43.2	36.8	42.7	38.0	44.6
TiO ₂	0.1	0.3			0.3	1.1	0.4	1.3
AlO _{1.5}	25.0	16.7	25.5	20.8	24.5	17.1	25.1	16.9
FeO	4.5	5.9			5.4	5.4	7.2	6.7
MgO	30.5	22.5	36.2	31.2	28.9	13.6	25.6	11.5
CaO	2.9	7.7	1.5	8.1	3.8	6.5	5.8	8.8
NaO _{0.5}		2.9			0.3	6.4	0.3	5.0
KO _{0.5}		0.1				1.9		0.5

Compositions of garnets (gt) and glasses (L) from experiments on the four mixtures used. Molar concentrations of each component are shown as the percentages of the total number of moles of all components as oxides. Mixture A was prepared from oxide powders to give garnets and melts similar to those found by Takahashi⁸ near the solidus of fertile peridotite at 3 GPa. Mixture B had the composition pyrope₈₀diopside₂₀, and was prepared from oxide powders. Mixtures C and D were made by adding 25% of the garnet composition as above to 75% of powdered ZION21, an alkali basalt from Utah (provided by J. G. Fitton) and MORB P22¹⁹ respectively. The compositions of the glasses and garnets in each experiment are available from the author on request. The experiments were conducted isothermally at near-liquidus temperatures such that only garnet and melt were present; the proportion of garnet was less than 25% in all experiments. The majority of garnet crystals analysed were smaller than 100 μm in diameter. Graphite capsules were used to produce a low oxygen fugacity and ensure that the uranium was dominantly tetravalent².

Garnet–melt pairs were produced by melting four compositions at pressures of 3–3.5 GPa. Mixture B is in the system CaO–MgO–Al₂O₃–SiO₂, mixtures A, C and D have the compositions of garnet plus basalt; A is the most basic, C and D give more evolved, alkaline melts. Between 0.05 and 0.2 mol% of UO₂, ThO₂, PbO, BaCO₃ and SrCO₃ were added in each experiment, except where stated. The experiments were conducted isothermally in graphite capsules to minimize the crystal growth rate (enhancing chemical equilibration at the garnet–melt interface) and to ensure that the uranium was dominantly tetravalent. Typical garnet and melt compositions are given in Table 1; the pressure, temperature and run duration of each experiment are shown in Table 2.

After quenching, up to five different crystals and glass areas in each charge were analysed by secondary ion mass spectrometry (SIMS) at the University of Edinburgh and by electron probe microanalysis (EPMA). The molar concentrations of each oxide component were calculated as percentages of the number of moles of all oxide components in each phase. Molar partition coefficients (D_*) for Sr, Ba, Pb, Th and U were calculated as the ratio of the mineral to melt oxide molar fractions, and are shown in Table 2. Errors quoted are propagated from the concentration range measured in each phase. Ba and Pb partition coefficients are close to background values and should be interpreted as upper limits. Experimental and analytical conditions were similar to those of ref. 2, except that talc-pyrex was used as the pressure medium in most experiments.

The high Th and U contents of the PB339 garnets and glass permitted analysis by electron microprobe. U and Th partition coefficients of $1.8 \pm 0.4 \times 10^{-2}$ and $1.9 \pm 0.6 \times 10^{-3}$ respectively were obtained using a 20-kV, 200-nA beam with 1,600-s counting times per element per spot. Thus the partition coefficients measured by SIMS and by EPMA are within two standard errors of each other, giving confidence in the analytical procedures used.

Three experiments were performed to test for deviations from Henry's law. Experiments PB301, PB319 and PB339 were all run at similar conditions using mixture A. All the measured partition coefficients for these experiments are identical within two standard errors, despite changes of up to two orders of magnitude in the concentrations of U, Th, Pb, Ba and Sr. If

TABLE 2 Experimental conditions and garnet-melt partition coefficients

	<i>T</i> (°C)	<i>P</i> (GPa)	Time (hours)	Mixture	<i>n</i>	$D_{Sr}^{gt/L}$ ($\times 10^{-3}$)	$D_{Ba}^{gt/L}$ ($\times 10^{-3}$)	$D_{Pb}^{gt/L}$ ($\times 10^{-3}$)	$D_{Th}^{gt/L}$ ($\times 10^{-3}$)	$D_U^{gt/L}$ ($\times 10^{-3}$)
PB167*	1,320	3.15	21.5	C	1	0.86	0.06	0.20	1.96	10.6
PB237†	1,450	3.0	27.2	A	3	0.85 ± 0.06	0.01 ± 0.16	—	3.26 ± 0.28	17.8 ± 2.7
PB300	1,565	3.1	24.0	B	3	0.48 ± 0.10	0.01 ± 0.04	0.67 ± 2.28	3.25 ± 0.97	17.9 ± 4.5
PB301	1,450	3.4	24.0	A	3	1.25 ± 0.57	0.04 ± 0.49	0.40 ± 0.53	2.49 ± 0.61	9.19 ± 1.4
PB306a‡	1,300	3.6	25.5	C	3	0.63 ± 0.01	0.01 ± 0.02	0.02 ± 0.03	1.59 ± 0.19	10.4 ± 1.3
PB306b‡	1,300	3.6	25.5	D	4	0.71 ± 0.05	—	—	1.52 ± 0.26	9.98 ± 1.7
PB319§	1,425	3.25	28.0	A	3	0.63 ± 0.04	0.01 ± 0.07	0.01 ± 0.03	1.50 ± 0.28	9.61 ± 1.26
PB339	1,425	3.25	28.0	A	5	1.00 ± 0.31	—	—	1.51 ± 0.82	9.18 ± 3.46

Experimental conditions, starting materials, and SIMS garnet-melt partition coefficients for Sr, Ba, Pb, Th and U (all $\times 10^{-3}$). *T*, *P* and time are the temperature, pressure and duration of each experiment; *n* is the number of garnet analyses per experiment. Molar partition coefficients (D_*) for Sr, Ba, Pb, Th and U were calculated as the ratio of the molar percentage of each oxide component in the garnet to that in the glass. The standard errors of the partition coefficients are calculated by propagating the variances of mineral and melt concentrations. Ba and Pb partition coefficients are close to detection limits, and should therefore be considered as upper limits. Only the lowest Ba and Pb partition coefficients for each experiment are shown in this table; standard errors calculated as for Sr, Th and U. The concentrations of SrO, BaO, PbO, ThO₂ and UO₂ in the melt phase were 0.05–0.2 mol% except as stated below.

* Only one analysable crystal found; 0.3–0.6 mol% SrO, BaO and PbO.

† Produced one very large crystal; data should be treated with caution as growth rates may have given rise to disequilibrium partitioning.

‡ Experiments run using BaCO₃ pressure medium, 0.3–0.6 mol% SrO, BaO and PbO.

§ 0.6 mol% SrO, BaO and PbO; 0.02% ThO₂ and UO₂.

|| 0.02% SrO, BaO and PbO; 1.5% ThO₂ and 0.1% UO₂.

Henry's law is satisfied over this large concentration range, then deviations from Henry's law between the concentrations present in these experiments and those occurring in the Earth's mantle are unlikely⁷.

The garnets and glasses produced in experiments with mixture A are similar to those near the solidus of fertile peridotite at 3 GPa (ref. 8). Thus the garnet-melt partition coefficients proposed for mantle melting are those of experiments PB319 and PB339: $D_{U}^{gt/L} \approx 9.6 \times 10^{-3}$, $D_{Th}^{gt/L} \approx 1.5 \times 10^{-3}$, $D_{Pb}^{gt/L} \approx 1 \times 10^{-5}$, $D_{Ba}^{gt/L} \approx 1 \times 10^{-5}$, $D_{Sr}^{gt/L} \approx 6 \times 10^{-4}$. $D_{U}^{gt/L}$ is much greater than $D_{Th}^{gt/L}$, in agreement with the Pb isotopic composition of garnets^{9,10}.

Assuming that $D_{U}^{cpx/L} = 9 \times 10^{-4}$, $D_{Th}^{cpx/L} = 1.3 \times 10^{-3}$, $D_{Ba}^{cpx/L} = 5 \times 10^{-4}$ and that olivine- and orthopyroxene-melt partition coefficients for U, Th and Ba are small², bulk partition coefficients can be calculated from the data above. For a garnet peridotite (gtpd) with 12% garnet, 8% clinopyroxene, 21% orthopyroxene and 59% olivine, $\bar{D}_{U}^{gtpd/L} = 1.2 \times 10^{-3}$, $\bar{D}_{Th}^{gtpd/L} = 2.9 \times 10^{-4}$ and $\bar{D}_{Ba}^{gtpd/L} = 4 \times 10^{-5}$. Thus for garnet peridotite, $\bar{D}_{U}^{gtpd/L} > \bar{D}_{Th}^{gtpd/L} > \bar{D}_{Ba}^{gtpd/L}$, in agreement with the order of U, Th and Ba concentrations in mid-ocean-ridge basalts (MORB) normalized to estimates of their asthenospheric concentrations¹¹.

Bulk partition coefficients for Th and U are small, and these elements will therefore behave incompatibly on melting: at high melt fractions very little Th or U will remain in the solid residue. Thus the (²³⁰Th/²³⁸U) systematics of MORB and ocean-island basalts will be dominated by mineral/melt partitioning on melt initiation. If melting were initiated in the spinel stability field then no (²³⁰Th) excesses would be produced, as U is more incompatible than Th during melting of spinel peridotite². By contrast (²³⁰Th/²³⁸U) ratios in excess of unity can be generated if melting is initiated in the garnet stability field, as $\bar{D}_{U}^{gtpd/L} > \bar{D}_{Th}^{gtpd/L}$.

The total melt fractions (*F*) for MORB and ocean-island basalts (OIB) are much greater than the bulk partition coefficients for Th or U; the observed excesses therefore cannot be produced by batch or rapid melting². Although the rare-earth element concentrations in the residues of mantle melting suggest that melting is nearly fractional⁴ (that is, the melt fraction in equilibrium with the solid during melting, ϕ , is small compared with the total amount of melt extracted), perfect fractional melting is not physically possible. If MORB and OIB form by melting on adiabatic decompression with near-constant upwelling velocity, then a dynamic melting model in which the

melting rate by mass (*M*) and the porosity (ϕ) are both constant will be a more appropriate model for melt generation. The equations for dynamic melting are close to those for fractional melting when $\phi \ll \bar{D}_*^{pd/L}$ and *F*.

The (²³⁰Th/²³⁸U) ratios produced by dynamic melting can be expressed as a function of ϕ and $\dot{M}$ (rearranged from equation 25 of ref. 12 and equation 1 of ref. 13)

$$\frac{(^{230}\text{Th})}{(^{238}\text{U})} = \frac{\bar{D}_{U}^{pd/L} + \Lambda}{\bar{D}_{Th}^{pd/L} + \Lambda} \quad (1)$$

where

$$\Lambda = \frac{\lambda_{230} \rho_L \phi + \dot{M}}{\lambda_{230} \rho_s (1 - \phi) - \dot{M}} \quad (2)$$

where λ_{230} is the decay constant for ²³⁰Th; ρ_L and ρ_s are the densities of the melt and of peridotite (2,800 and 3,300 kg m⁻³ respectively). It is assumed that the time between magma generation and eruption is short compared with the half-life of ²³⁰Th

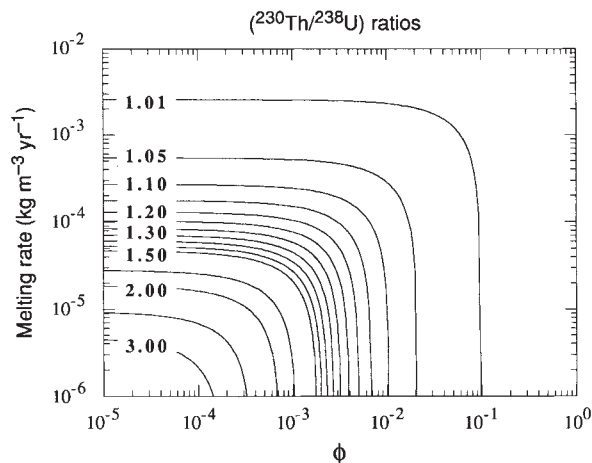


FIG. 1 (²³⁰Th/²³⁸U) ratios produced on partial melting of garnet peridotite as a function of melting rate (*M* in kg m⁻³ yr⁻¹) and porosity (ϕ). The generation of (²³⁰Th/²³⁸U) values of 1.25, as observed in recent mid-ocean-ridge basalts and most recent ocean-island basalts, require porosities on melting of less than 4×10^{-3} , and melting rates slower than 10^{-4} kg m⁻³ yr⁻¹. These data are consistent with mantle upwelling velocities of the order of 0.01 m yr⁻¹, as assumed in ref. 20.

(75,000 years). As can be seen from equation (1), (^{230}Th) excesses require that $\bar{D}_{\text{U}^*}^{\text{pd/L}}$ is greater than $\bar{D}_{\text{Th}^*}^{\text{pd/L}}$ on melt initiation, which will be satisfied if melting is initiated in the garnet stability field.

Infinitely slow fractional melting of peridotite in the garnet stability field, instantaneously extracted, would yield $(^{230}\text{Th}/^{238}\text{U}) = \bar{D}_{\text{U}^*}^{\text{pd/L}} / \bar{D}_{\text{Th}^*}^{\text{pd/L}} \approx 4$. Where melting is more rapid, the porosity non-zero, or transfer to the Earth's surface not instantaneous, the observed $(^{230}\text{Th}/^{238}\text{U})$ will approach unity. Fig. 1 shows the (^{230}Th) excesses that will be produced by melting initiated in the garnet stability field as a function of $\bar{M}$ and ϕ , calculated using equation (1) and the bulk partition coefficients for garnet peridotite calculated above: (^{230}Th) excesses are produced when both $\bar{M}$ and ϕ are small.

The $(^{230}\text{Th}/^{238}\text{U})$ ratios of recent fresh MORBs vary from 0.93 to 1.6, and cluster around 1.25 (refs 14–18). The generation of $(^{230}\text{Th}/^{238}\text{U})$ ratios of 1.25 requires that the porosity is less than 4×10^{-3} , even if melting is infinitely slow. Thus mid-ocean-ridge melting is near-fractional, and the small range in the compositions of MORB therefore requires that efficient magma mixing occurs on ascent. Evident for such magma mixing can also be seen in the chemistry of MORB¹⁹.

The (^{230}Th) excesses of MORB also require that the melting rate at the solidus must be less than $10^{-4} \text{ kg m}^{-3} \text{ yr}^{-1}$, or smaller for non-negligible porosities. Mass balance requires that the upwelling rate $W \approx \bar{A}\bar{M}/F\rho_L$ if the melting rate is roughly constant at all melt fractions. Hence $W \approx 100 \bar{M} \text{ m yr}^{-1}$ for magma of density $\rho_L = 2,800 \text{ kg m}^{-3}$ produced on melting by $F = 20\%$ over a thickness A of 50 km. Thus average melting rates of less than $10^{-4} \text{ kg m}^{-3} \text{ yr}^{-1}$ are compatible with upwelling velocities of the order of 0.01 m yr^{-1} , as assumed in ref. 20. Such upwelling velocities are smaller than the half spreading velocity for the East Pacific Rise (0.03 m yr^{-1} ; ref. 21) and require that mantle upwelling is distributed over tens of kilometres either side of the ridge axis. This constraint would be relaxed if the melting rate increased with melt fraction.

Recent basalts from most ocean islands yield $(^{230}\text{Th}/^{238}\text{U})$ ratios of between 1.1 and 1.5 (refs 22–26), demonstrating that the melting rates are less than $3 \times 10^{-4} \text{ kg m}^{-3} \text{ yr}^{-1}$. By contrast, all but six of the 50 published $(^{230}\text{Th}/^{238}\text{U})$ ratios for Hawaii are within two standard deviations of unity^{24,25,27–29}. The lack of (^{230}Th) excesses at Hawaii may be due to the large upwelling rate of its mantle plume (0.3 m yr^{-1})³⁰. Thus the $(^{230}\text{Th}/^{238}\text{U})$ ratios of MORB and most OIB can be produced if melting is initiated in the garnet stability field, and magma is generated slowly and at low porosities. □

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Exploitation of cold temperature as defence against parasitoids in bumblebees

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PARASITES can modify host behaviour for their own benefit after infection^{1–4}. Whether these changes also minimize loss of host fitness is less well known^{5–7}. Although parasitoids are abundant⁸, few cases are known of behavioural changes in host-parasitoid systems⁹. Bumblebees (*Bombus* spp., Apidae, Hymenoptera) are primitively eusocial insects with an annual life cycle. Reproduction occurs in late summer when males and young queens are released^{10,11}. Parasitoid conopid flies (Conopidae, Diptera) are abundant in summer, and up to 70% of field-caught bumblebee workers may be parasitized¹². Development of the parasitoid usually takes 10–12 days and ends with the host's death when the larva pupates. Here we report a novel strategy used against these parasitoids, based on the exploitation of temperature effects on parasite development. We demonstrate that parasitized workers of the bumblebee *Bombus terrestris* L. stay in the field overnight rather than returning to their nest. This behaviour retards the development of the parasite and reduces the chances of successful development. In choice experiments, parasitized foragers actively seek out cold temperatures. This behaviour therefore enhances colony success by prolonging the life of parasitized workers while at the same time reducing parasitoid fitness.

Bumblebee workers parasitized by conopid larvae (the flies oviposit an egg into the bee's abdomen) often stay away from their colonies at least during daylight foraging hours¹³. This seems a paradoxical behaviour, for workers of social insects only gain reproductive success by helping their mother-colony to rear offspring. But colony desertion could benefit the host if the thermal environment of a developing parasitoid larva thereby deteriorates, because development in insects is temperature-dependent¹⁴. If parasitized workers also stay outside the nest at night, and thus experience low temperatures, this would retard the development of the parasitic larva and prolong host lifespan. In contrast, workers that stay out in the field during the day, or inside the nest at night, experience temperatures near 30 °C, for the brood is actively incubated¹¹. To investigate this, we first established 29 colonies of *B. terrestris* in the field. Colonies were censused every night and all workers were individually marked.

TABLE 1 The number of parasitized and nonparasitized foragers that spent the night inside or outside the nest

	Foragers found inside colony at night	Foragers not returning until next morning
Nonparasitized	74 (61.8)	90 (102.2)
Parasitized	4 (16.2)	39 (26.8)

Parasitized foragers were more likely to remain outside their colony at night ($\chi^2 = 18.61$, $n = 207$, $P < 0.0001$). Numbers in parentheses are expected values.

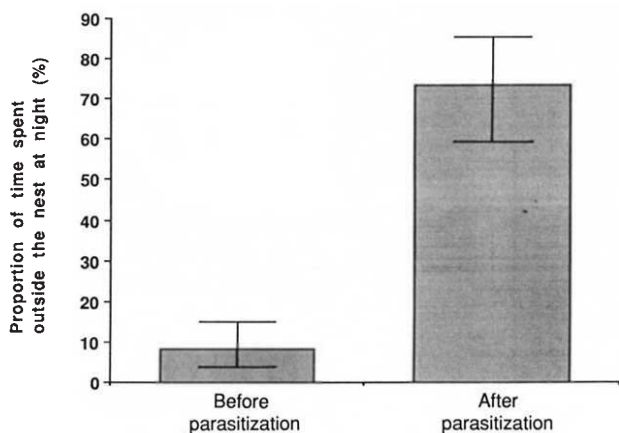


FIG. 1 Known foragers of *B. terrestris* colonies stayed outside the nest 73.5% of the nights after becoming parasitized, significantly more often than the 8.4% of nights before their parasitization (paired $t=9.59$, d.f. = 64, $P=0.0001$). A total of 65 foragers were followed. The date of infection was estimated by subtracting 1 day for the time of oviposition for the egg stage, 3 days for the 1st instar, 6 days for the 2nd instar, 8 days for a 3rd instar, and 10 days for pupa, depending on the actual stage of the parasite at collection of the forager (for developmental times: R. Schmid-Hempel, manuscript in preparation). To exclude the possibility that the increase shown in the graph is a simple effect of worker age, we calculated logistic regression coefficients for the proportion of time spent outside by each forager as a function of its parasitization (yes/no) and its age for randomly chosen subsets of the data. Parasitization turned out to be significant in 8 out of 10 runs but age only in 1 out of 10, suggesting that age indeed plays a minor role. No effect of colony identity was found. Bars in the graph denote 95% confidence interval.

From July to mid-August, we recorded the identity of the foragers, and each week we removed 2–3 known foragers from the nests at night to check for parasitization by conopid flies. In addition, traps were installed to catch the workers that had spent the night outside and were returning to the nest to resume foraging the next morning. The traps were left in place until noon, and these colonies were supplemented with pollen and diluted honey for the loss of normal foraging. All workers collected in the traps were dissected and checked for parasitoids.

Among the foragers collected from inside the nest at night, only 5.4% were parasitized, significantly fewer than the 43.3% parasitized foragers that were trapped returning to the nest the following morning (Table 1). This difference resulted from parasitization, rather than from an overall tendency of these individuals to stay outside or from a simple age effect (Fig. 1). To test further whether cold temperatures affect parasitoid development and lifespan of parasitized workers, 304 foragers were captured in the field and assigned at random to one of four treatments: an ambient temperature of 19 °C or 28 °C combined with either constant darkness or a 16:8 h light/dark cycle. Temperatures were chosen to reflect the average experienced by workers staying outside in the field during summer nights or inside the colony, respectively. The light cycle controlled for the possibility that light and temperature might interact in affecting parasitoid development, such that bees increase their day-light activity levels to compensate for low temperatures. The results demonstrated that low ambient temperature had both a positive effect on host worker longevity and a negative effect on size of the larval parasitoids (Fig. 2). There was no significant interaction between temperature and light cycle for worker longevity or parasitoid development. At host death, parasitoids had successfully completed their development in 48 cases in cold temperatures, but in 44 cases the parasite was still too young to pupate. In warm temperatures, in contrast, significantly more parasites completed development (87 cases versus 18 cases) ($\chi^2=21.4$, $n=197$, $P<0.001$). Hence, cold ambient temperature prolonged host lifespan by reducing developmental rates of the

parasitoid and by reducing its ability to pupate successfully. In a final experiment, we tested whether parasitized foragers actively prefer cold temperatures. A total of 61 foragers were supplied with diluted honey and could move freely between a cold and a warm compartment. The location of each worker was recorded several times per day and after the worker's death parasitization was assessed. Thus parasitized workers spent significantly more time in the cold area than did nonparasitized workers and thus they actively seek out a cold environment (Fig. 3).

Our experiment has addressed the adaptive value of the behavioural change. Here we demonstrate that by exposing themselves to cold temperatures during times when foraging is impossible anyway (that is, at night) parasitized bumblebee workers prolong their foraging career to work for the colony while negatively influencing parasitoid development and success. Host advantage and behavioural options available for social insects can thus explain the paradoxical desertion

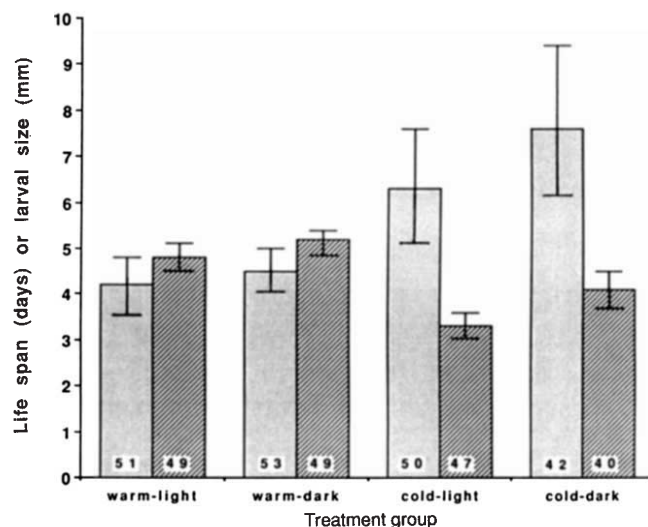


FIG. 2 Parasitized foragers were kept under different temperature ('warm' = 28 °C, 'cold' = 19 °C) and light regimes ('dark' = no light, 'light' = 16:8 h day/night cycle) in separate containers (55 × 35 × 40 cm). Diluted honey was supplied and the containers were checked for dead bees three times a day. All workers were dissected after death and the size of the parasitoid larvae was recorded. A total of 196 foragers was tested. Lifespan refers to residual life time after the start of the experiment. Lifespans were significantly affected by temperature but not by light cycle or interaction (ANOVA, d.f. = 192, temperature $F=29.3$, d.f. = 1, $P=0.0001$; light $F=2.16$, d.f. = 1, $P=0.14$; temperature × light $F=0.55$, d.f. = 1, $P=0.46$). Also, larval sizes showed a temperature effect and no interaction (ANOVA, d.f. = 181, temperature $F=62.41$, d.f. = 1, $P=0.0001$; light $F=11.32$, d.f. = 1, $P=0.001$; temperature × light $F=2.74$, d.f. = 1, $P=0.1$). Analyses were done with $\ln(x+1)$ -transformed data. Bars denote 95% confidence intervals, numbers in bars are sample sizes.

behaviour. But this cannot explain the underlying mechanism that causes altered behaviour. As yet, we can only hint at some possibilities. For example, bumblebees parasitized by conopid flies have a high incidence of hypothermia, even while foraging¹⁵. Hence, parasitized bees may be more likely to become torpid when foraging late in the day as ambient temperature decreases. Alternatively, parasitized workers may experience sensory impairment and fail to locate the nest opening¹⁶.

Bumblebees are well adapted to cold climates and can fly at low ambient temperatures. This is achieved with massive heat production of the flight muscles that leads to thoracic temperatures over 30 °C maintained against large thermal gradients¹¹. For such a heterothermic animal, reduction of body temperature thus requires a colder ambient environment.

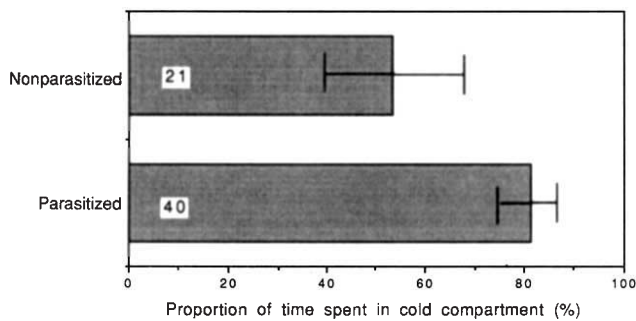


FIG. 3 In a choice experiment, 61 bees could move freely between two compartments ($40 \times 35 \times 40$ cm) of different average ambient temperatures (17.2 ± 2.4 °C, and 29.2 ± 1.7 °C, respectively). The proportion of time spent in the cold compartment was significantly higher for parasitized bees than for nonparasitized ones ($t=4.1$, d.f.=59, $P=0.0001$, arcsin-transformed data). Bars are 95% confidence intervals, numbers in bars are sample sizes.

Increasing body temperature is a strategy to combat parasitic infections in vertebrates¹⁷ and some insects^{18,19}. The case reported here demonstrates for the first time that a comparable

strategy, based on altered behaviour that places parasitized individuals into cold environments, is used against parasitoids by an insect host. □

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Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants

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AMONG the Eukaryota, the true fungi comprise four divisions (Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota) that constitute a natural group which is thought to have diverged about 1 billion (10^9) years ago, believed also to be the time of divergence between metaphyta and metazoa lineages¹. The endosymbionts responsible for the most prevalent plant root symbiosis, the vesicular–arbuscular mycorrhizae (VAM) or, more appropriately, arbuscular mycorrhizae, comprise 130 species of fungi classified in the Zygomycotina, order Glomales^{2–4}. The arbuscular endomycorrhizae are considered to be ecologically important for most vascular plants^{5–8} in view of their beneficial effects on plant growth and survival. They are one of the few plant–fungus associations with a fossil record and may even have facilitated the origin of land flora. But the biochemical and genetic characterization of these microsymbionts has been hampered by the inability to grow them in pure culture. To investigate the origin and clarify the phylogenetic relationships of these organisms, we have sequenced ribosomal DNA genes from twelve species. Our phylogenetic analyses confirm the existence of three families within arbuscular fungi on the basis of morphological characters. We obtain approximate dates for the divergence of major branches on the phylogenetic tree. These include an estimate for the origin of VAM-like fungi of 353–462 Myr ago, which is consistent with the hypothesis that VAM were instrumental in the colonization of land by ancient plants.

Because of our inability to grow VAM fungi in culture, they have obligatory biotrophic status. Consequently, the taxonomy of these fungi has been largely based on the morphology of the large soil-borne spores, typically 80 to 500 μ m in diameter, which

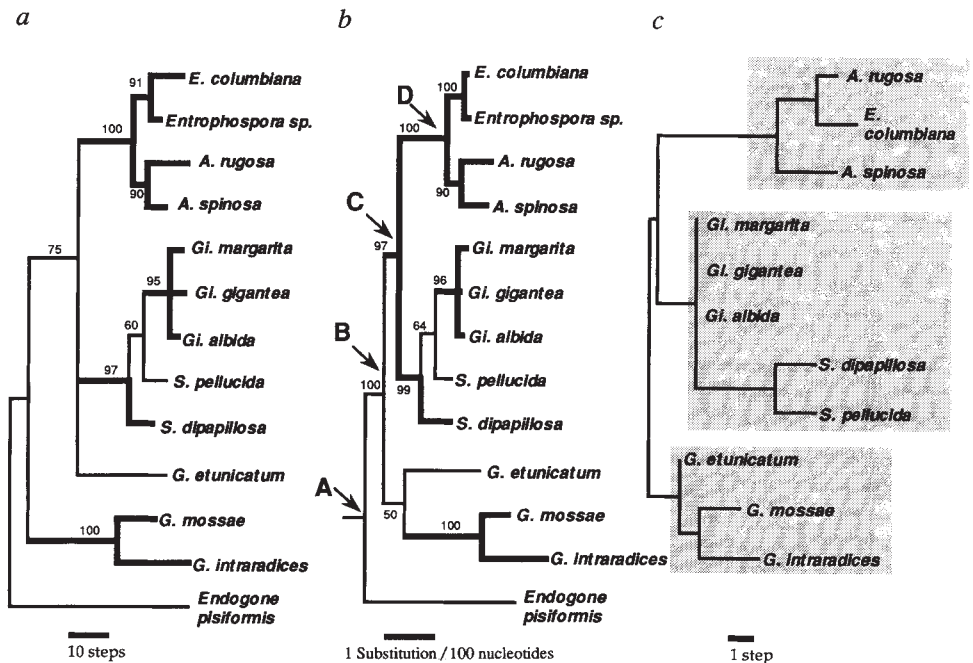
can be found near colonized roots. To study their phylogeny and evolutionary history, our approach was to amplify and sequence the nuclear genes encoding their small-subunit rRNA (SSU), out of a few spores.

Twelve species of VAM fungi representative of the main taxonomic groups recognized at present were analysed. Almost complete sequences of the SSU were obtained by direct sequencing of both strands of the polymerase chain reaction (PCR) products. Comparison of arbuscular endomycorrhizal SSU sequences with those of other fungi and fungal-like eukaryotes had already confirmed their classification within the true fungi⁹. Both parsimony and distance matrix analysis support the monophyletic origin of the VAM. The revised morphologically based classification of arbuscular mycorrhizal fungi into three families, the Glomaceae, Acaulosporaceae, and Gigasporaceae, is supported by our molecular analysis (Fig. 1a, b). The three *Gigaspora* species analysed had few differences in their SSU sequences, and were shown to have diverged from *Scutellospora*, which contradicts the morphological notion that *Gigaspora* are less evolved than *Scutellospora*⁴. In addition, unexpectedly large differences were observed among the three representatives of the Glomaceae: for instance, more differences were observed between *G. etunicatum* and either *G. mossae* or *G. intraradices* than could be seen between Acaulosporaceae and Gigasporaceae. This explains its equivocal placement in Fig. 1a and b, and suggests that the Glomaceae could possibly be subdivided as more species are sequenced.

We conducted Wagner parsimony analysis of a set of 27 morphological characters compiled for 11 of the 12 arbuscular mycorrhizal fungi analysed for SSU sequences⁴. Five of the 27 characters were invariant, seven were uninformative, and 15 were informative. A topology very similar to that obtained with DNA sequence data was recovered (Fig. 1c).

Fossils recently discovered in Triassic sediments from Antarctica indicate that arbuscular mycorrhizal fungi were well established by the early Mesozoic (215 Myr)¹⁰, and a reappraisal of Rhynie Chert plants from the Devonian suggests that primitive plants might already have been associated with arbuscular mycorrhizal fungi between 410 and 360 Myr ago¹¹. As only *Glomus*-like fossil fungi have been reported, nothing is known about the evolutionary history leading to the extant arbuscular mycorrhizal fungi families. SSU sequences were used as a molecular clock to infer dates of divergence between the major clades in the phylogenetic tree of the Glomales.

FIG. 1 Phylogenetic tree of VAM fungi. Spores of the different species were used as starting material for the amplification of the nuclear gene coding for the small subunit rRNA (SSU). Preparation of crude extracts, amplification, preparation, and sequencing of single-stranded templates has been described¹⁶. Alternatively, double-stranded templates were sequenced using a cycle-sequencing protocol (Dye-Deoxy sequencing kit, ABI, Ontario). The sequencing reactions were subsequently loaded on a 373A fluorescent sequencer (ABI). SSU sequences were obtained for 10 arbuscular fungi (accession numbers: *Glomus etunicatum*, Z14008; *G. mossae*, Z14007; *Entrophospora* sp., Z14011; *E. columbiana*, Z14006; *Acaulospora spinosa*, Z14004; *A. rugosa*, Z14005; *Gigaspora gigantea*, Z14010; *Gigaspora albida*, Z14009; *Scutellospora pellucida*, Z14012; *S. dipapillosa*, Z14013). These sequences were compared with published VAM fungi SSUs and with other eukaryotic SSUs. Gaps were manually inserted to optimize the alignment. Whenever possible, secondary structure features were used to position the gaps better. Of the 1,909 positions in the dataset, 450 whose alignment was questionable were not considered in the analyses. *a*, A subset of the dataset comprising sequences from VAM fungi and *Endogone pisiformis* as outgroup was selected. Parsimony analysis was conducted with PAUP v.3.0s (ref. 17), using the Branch and Bound algorithm, and bootstraps estimated from 600 replicates. The consensus tree is depicted; thick lines delineate the topology that is supported by 90% or more of the bootstraps. One most parsimonious tree of length 232, excluding the outgroup, was obtained; consistency index, 0.746. Numbers on branches indicate per cent bootstrap estimates. *b*, Similar topology and confidence were obtained by calculating pairwise substitution rates using a one-parameter



eter method¹⁸ and submitting the distance matrix to the neighbour-joining method of tree construction¹⁹. The root of this tree was established by including sequences from plants and *Stylonychia* in the analysis. Bootstraps were obtained from 200 replications using the NJBOOT program. Lettered arrows indicate events considered in the estimation of divergence times. *c*, Topology of the shortest mid-point rooted tree obtained by Wagner parsimony analysis of morphological data in ref. 4, with length 32 and consistency index 0.938. Shaded boxes delineate the three families of VAM fungi: Acaulosporaceae (top), Gigasporaceae (middle) and Glomaceae (bottom).

As a prerequisite to the construction of clocks, homogeneity of substitution rates was assessed using a lineage relative rate test¹². No significant rate heterogeneity was detected within the Glomales, as well as between the plant and Glomales, although the rate was 15 per cent slower in the latter, so that the divergence dates within the fungi could be slightly underestimated. Two calibration points were used to obtain substitution rates per site per year. These rates were used to translate half of the mean number of substitutions between any two clades into time since divergence. The first calibration was 200 Myr for the split between monocot and dicot plant lineages¹³. The second milestone was 1,000 Myr for the radiation of major eukaryotic clades, including fungi and metaphtya¹. Our analysis placed the origin

of VAM fungi between 462 and 353 Myr ago, a period delineated by the estimates obtained for the divergence between Glomales and a non-VAM zygomycete (*Endogone pisiformis*), and the estimates for the divergence between the Glomaceae and the other arbuscular fungi (Fig. 2). The families Acaulosporaceae and Gigasporaceae appeared later and diverged from each other in the Late Palaeozoic, 250 Myr ago. The split between *Entrophospora* and *Acaulospora* occurred during the Cretaceous. Based on these results, it could be deduced that ancestral endomycorrhizal fungi were probably *Glomus*-like and originated in the Palaeozoic era, possibly at the time of appearance of the first land plants (415 Myr)¹⁴. This is in agreement with the fossil record, as exemplified by the Rhynie Chert plants

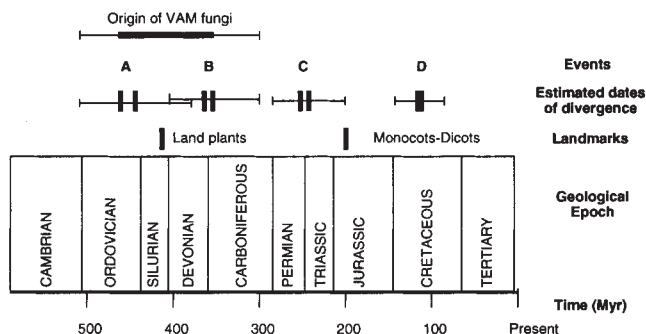


FIG. 2 Estimated dates of origin and divergence of VAM fungi. Using aligned SSU gene sequences of four angiosperms (dicots: *Alnus glutinosa* and *Lycopersicon esculentum*; monocots: *Oryza sativa* and *Zea mays*) in addition to the VAM fungi sequences, the number of substitutions per site were calculated using a one-parameter method¹⁸. Results obtained with a two-

parameter method²⁰ were essentially the same and are not presented. Before calibrating the clock, a lineage relative rate test was performed¹², with the protist *Stylonychia* as reference taxon. No significant difference (at $P < 0.01$) was found between substitution rates of plants and fungi, between Glomaceae and other VAM fungi, between Acaulosporaceae and Gigasporaceae, or between Acaulosporae and Entrophosporae. Two estimates of substitution rate per year were obtained: (1) by dividing half the average number of substitutions per site between the dicots and the monocots (0.026687) by 200 Myr (ref. 13); (2) by dividing half the average number of substitutions per site between the angiosperms and the fungi (0.128301) by 1,000 Myr (ref. 1). Divergence times were estimated for the nodes indicated on Fig. 1b by applying both substitution rates per year (6.67×10^{-11} between dicots and monocots; 6.42×10^{-11} between plants and fungi) to half of the average number of substitutions between lineages, calculated as the mean of all relevant pairwise comparisons between taxa: A, 444–462 Myr; B, 353–367 Myr; C, 243–252 Myr; D, 113–117 Myr. Error estimates were computed for each divergence date by adding both the standard deviation calculated from the Jukes–Cantor distance¹⁸ used to estimate substitution rates per year, and the standard deviation from the mean distance between lineages.

from the Devonian such as *Aglaophyton*, *Rhynia* or *Asteroxylon*, which contain structures interpreted to be vesicles and spores resembling those formed by extant *Glomus*¹¹. Our dating of the origin of VAM fungi is consistent with the hypothesis that symbiotic fungi were instrumental in the colonization of land by ancient plants¹⁵. This hypothesis is also supported by the observation that endomycorrhizae can now be found worldwide on most extant plant families, in the angiosperms, gymnosperms as well as vascular cryptogams (ferns), suggesting that its nature is ancestral. □

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Transgenic *N. glauca* plants expressing bacterial virulence gene *virF* are converted into hosts for nopaline strains of *A. tumefaciens*

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TUMOURS are induced by *Agrobacterium tumefaciens* on a variety of plants^{1–3}. The virulence determinants of *A. tumefaciens* reside on a large tumour-inducing (Ti) plasmid. This plasmid carries two regions essential for tumour induction, namely the T region and the Vir region. During infection the T region is transferred to the plant cell, where it becomes stably integrated in one of the host chromosomes as T-DNA. Expression of T-DNA leads to the production of the plant hormones auxin and cytokinin, as well as to the synthesis of specific amino-acid derivatives termed opines. *Agrobacterium* strains are classified according to the types of opines produced by the tumours they induce. The Vir region contains genes that are expressed in the bacterium and are required for T-DNA transfer to plant cells, and several other genes that affect the efficiency of transfer and the host range. Vir regions from different Ti plasmids may vary slightly in the genes they contain: for instance, the *virF* gene, which is present in the Vir-region of octopine Ti plasmids, is absent from nopaline Ti plasmids^{4,5}. Mutation of the *virF* gene leads to a weakened virulence of octopine strains on tomato⁶ and *Nicotiana glauca* (shrub tobacco)⁴. Nopaline strains are strongly attenuated in *N. glauca* compared with octopine strains because of the absence of the *virF* virulence gene from the Ti plasmid in nopaline strains⁴. The *virF* gene product may be transferred to and be active in plant cells. Here we isolate transgenic *N. glauca* plants in which the *virF* coding sequence is expressed using the cauliflower mosaic virus 35S promoter. The presence of the VirF protein converts the non-host *N. glauca* into a host for tumour formation by *A. tumefaciens* nopaline strains and octopine *virF* mutants. Our results indicate that certain virulence gene products such as the VirF protein may be transferred to plant cells during tumour induction, where they function as mediators of T-DNA transfer.

In contrast to other *vir* mutants, strains defective in *virE* or *virF* can be complemented for tumorigenicity by coinfection

with a helper strain such as LBA 4404, which does not carry a T region and so is not oncogenic, but which has an intact octopine-type Vir region^{5,7}. Helper strains in which one of the essential *vir* genes is defective cannot complement *virE* and *virF* mutants for tumour induction^{4,7}, indicating that a complete *vir*-encoded (T-DNA) transport system is necessary for complementation by co-infection. As there is no T-DNA transfer from these helper strains, certain Vir proteins or their products may be transferred to plant cells by means of this *vir*-encoded

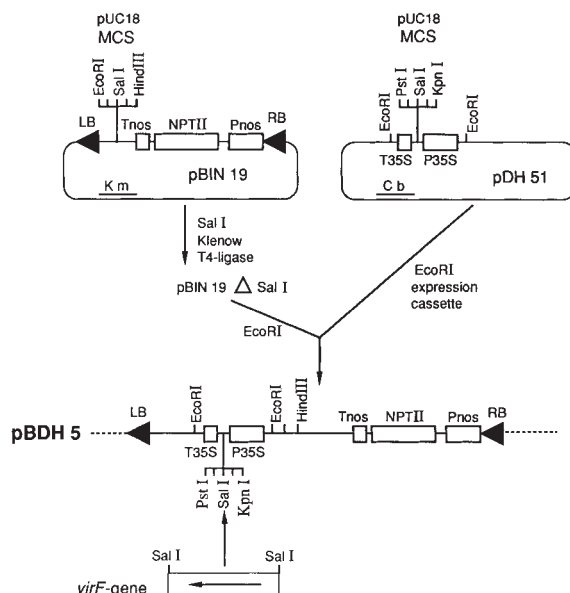


FIG. 1 Construction of a vector for expressing the VirF protein in plants. The plant expression vector pBDH5 present in *E. coli* CEL475 (ref. 13) was constructed by deleting the *SalI* restriction site from pBIN19 (ref. 14) and subsequently inserting a CaMV 35S expression cassette from pDH51 (ref. 15) into the *EcoRI* site. To clone the coding region of the *virF* gene as a *SalI* fragment in pBDH5, it was first subcloned as a *BspHI* fragment from plasmid pRAL7007 (ref. 4), which is a derivative of pIC19R (ref. 16) with an *SstI* fragment containing the *virF* gene, into the *NcoI* site of pMTL24 (ref. 17). The resulting construct was termed pRAL7014. Plasmid pRAL7014 was isolated from *E. coli* and electroporated into the *A. tumefaciens* helper strain LBA4404 (ref. 18). Cloning procedures were according to ref. 19; electroporation of agrobacteria was according to ref. 20. Abbreviations: MCS, multiple cloning site; LB, RB, left and right T-DNA border repeats; T35S and Tnos, cauliflower mosaic virus (CaMV) 35S and nopaline synthase terminator sequences; P35S and Pnos, CaMV 35S and nopaline synthase promoter sequences; NPTII, neomycin phosphotransferase gene; Km, bacterial kanamycin-resistance selection marker; Cb, bacterial carbenicillin-resistance selection marker.

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and protein may have a common evolutionary origin, especially considering that it is not naked DNA that is transported but a pilot protein (VirD2 in the case of T-DNA transfer) with a covalently attached DNA strand¹⁻³. □

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Co-existence of cationic and chloride components in odorant-induced current of vertebrate olfactory receptor cells

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ODORANT stimulation leads to a depolarization of olfactory receptor neurons¹⁻³. A mechanism underlying this transduction, which occurs in the sensory cilia³⁻⁶, involves a G-protein-mediated increase in adenylyl cyclase activity⁷⁻¹⁰, and therefore a rise in internal cyclic AMP and consequent opening of a cAMP-gated cation channel on the plasma membrane¹¹⁻²². Another mechanism, not as well established, involves the opening of an inositol triphosphate-activated cation channel on the plasma membrane²³ as a result of phospholipase C activity^{24,25}. In both cases, an influx of cations is thought to generate the depolarizing receptor potential. We now report, however, that the mechanism is actually more complex. The odorant-induced current appears to contain an inward chloride component also, which is triggered by calcium influx through the cation-selective channel. This newly found chloride component can be as large as the cationic component. The co-existence of cationic and chloride components in the odorant response, possibly unique among sensory transduction mechanisms, may serve to reduce variations in the transduction current resulting from changes in external ionic concentrations around the olfactory cilia. Our finding can explain the long-standing puzzle of why removal of most mucosal cations still does not diminish the amplitude of the olfactory receptor cell response²⁶⁻²⁸.

Whole-cell recordings under voltage-clamp were made from single, dissociated olfactory receptor neurons of the newt (*Cynops pyrrhogaster*) while brief pulses of odorant (a mixture of *n*-amylacetate, isoamylacetate and limonene) were applied through a puffer pipette (for methods, see Fig. 1 legend). Similar experiments on adult tiger salamander (*Ambystoma tigrinum*) olfactory neurons gave identical results.

The involvement of a Cl^- conductance in olfactory transduction was first suggested by the effect of external Na^+ concentration, $[\text{Na}^+]_o$, on the reversal potential of the odorant-induced current. In physiological saline, odorant stimulation elicits current responses that are monophasic at all voltages and have a reversal potential near +5 mV (Fig. 1a). In the absence of

external Ca^{2+} , this reversal potential depends strongly on external Na^+ (ref. 3); it shifts negatively by ~57 mV for a 10-fold reduction in $[\text{Na}^+]_o$ (Fig. 1c, solid line), consistent with the opening of a cation-selective conductance and the near-bicubic condition of such an experiment (external Na^+ and internal Cs^+ , with isotonic Cl^- on both sides of the membrane; Fig. 1 legend). In the presence of external Ca^{2+} , however, the reversal potential became quite insensitive to $[\text{Na}^+]_o$ and remained near 0 mV even with very low external Na^+ (Fig. 1b, and dashed line in Fig. 1c), suggesting that there is another major current component in the olfactory response. This component cannot be carried by Ca^{2+} , because Ca^{2+} is not a major current carrier through the odorant-induced cation conductance^{3,29}. It must be triggered instead by a rise in intracellular Cs^{2+} , most probably a Ca^{2+} -activated Cl^- current; a Ca^{2+} -activated K^+ current would have been blocked by the internal Cs^+ used in these experiments, or otherwise would have given a very negative reversal potential from 0 mV. The existence of a Ca^{2+} -activated Cl^- conductance has very recently been reported in frog olfactory cilia³⁰.

To check the involvement of chloride, we reduced the external Cl^- concentration, $[\text{Cl}^-]_o$, to 34 mM by replacement with gluconate. In this case, the odorant-induced current became conspicuously biphasic at +24 mV, consisting of an outward component followed by an inward component (Fig. 2a). The current-voltage relation measured at two different time instants during the response is shown in Fig. 2b. The positive shift of the reversal potential at later time (t_2) is consistent with a delayed Cl^- current subject to an equilibrium potential which is very positive from zero owing to the low $[\text{Cl}^-]_o$. All cells tested ($n = 10$) showed similar results, suggesting that the Cl^- current is probably present in all receptor cells.

The presence of a chloride component in the olfactory response was further confirmed using the chloride-channel blocker SITS, which selectively removed the inward current in the biphasic odorant response elicited in low external Cl^- and at a positive membrane potential, as already described (Fig. 3a). The effect of SITS was rapid and reversible. This drug had little effect on the odorant response in the absence of external Ca^{2+} , indicating that it blocked the Cl^- component specifically (data not shown). The time courses of the cationic (trace a) and Cl^- (trace b; obtained by subtraction, see legend) currents from the experiment shown in Fig. 3a are plotted in Fig. 3b. The Cl^- current was more prolonged; it was also activated more slowly than the cationic current, consistent with its induction by a Ca^{2+} influx through the cation-selective channel. Figure 3c shows a similar experiment, except in this case the cell was bathed in physiological saline and the membrane potential was held at -54 mV, close to the physiological resting potential of these cells. 2 mM SITS again reduced the amplitude of the odorant-induced inward current, with the separated cationic (trace a) and Cl^- (trace b) components being shown in Fig. 3d. From a

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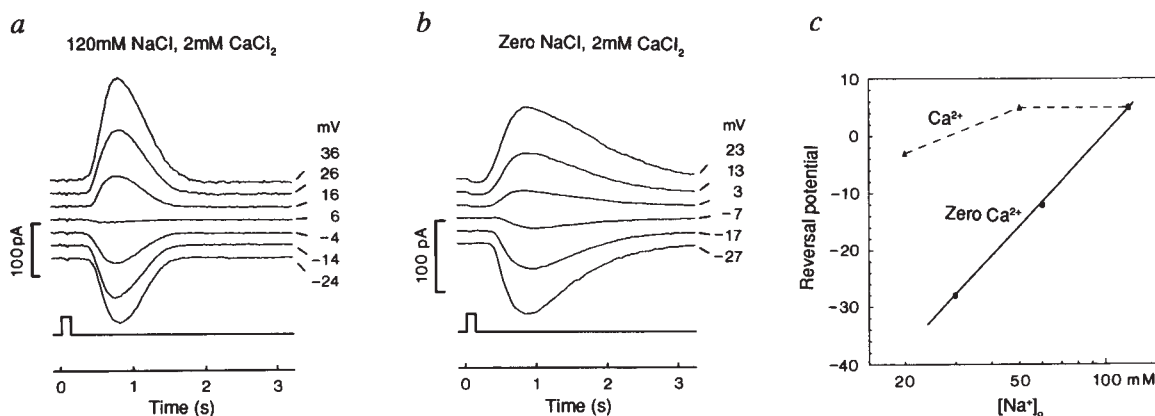


FIG. 1. *a*, Voltage dependence of the odorant-induced current recorded from a newt olfactory receptor neuron. The levels of the current traces have been displaced from each other for clarity. Upward direction on the ordinate indicates outward membrane current. Timing of odorant stimulation is indicated by the upward deflection shown below the current traces. In this experiment the cell was bathed in physiological saline solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 Na-HEPES, 10 glucose, pH 7.4). Whole-cell recording pipette contained (in mM): 120 CsCl, 5 Na-EGTA, 10 Na-HEPES, pH 7.4. *b*, Odorant-induced currents in low-Na⁺ external solution; newt receptor cell is different from *a*. The bath solution contained (in mM): 120 choline-Cl, 2 CaCl₂ and 10 Na-HEPES, pH 7.4; same pipette solution as in *a*. In both *a* and *b*, each trace represents the response to a single stimulus trial. *c*, Na⁺ dependence of the reversal potential of the odorant-induced current in the presence (3 mM; triangles) and absence (circles) of external Ca²⁺; Na⁺ replacement by choline. The data for zero Ca²⁺ are redrawn from ref. 3. Each point represents the average from at least 2 different cells. The solid line has a slope of 57 mV per 10-fold change in [Na⁺]_o. Data are from newt.

METHODS. The animal, newt or salamander, was pithed and the dorsal bone covering the olfactory cavity removed. The olfactory epithelia were excised and then dissociated into individual cells as described³. Briefly, epithelia

were cut into pieces of 4–9 mm² and treated with 0.1% collagenase (Sigma; type 1A) for 5 min at 37 °C in normal saline of composition as described, except CaCl₂ and MgCl₂ were omitted. After rinsing several times with normal saline, epithelial pieces were dissociated by trituration with a Pasteur pipette. Dissociated cells were plated onto concanavalin A-coated culture dishes and stored under normal saline at 4 °C for up to 10 h. Cells were scanned on a microscope with phase-contrast optics and recordings were made with the whole-cell patch clamp method. The patch pipette had a tip opening of ~0.5 µm in diameter and a resistance of ~10 Mohm. Seal resistance upon establishment of a membrane patch was ~10 Gohm. The bath electrode consisted of a Ag-AgCl wire connected to the bath solution via an agar bridge (120 mM NaCl). Change in bath solution took ~2 s to complete. Junction potential changes at the tip of the agar bridge were measured with a microelectrode containing saturated KCl; all data reported here have been corrected for junction potentials. The odorant used for stimulation consisted of a mixture of *n*-amylacetate (1 µl ml⁻¹), *iso*-amylacetate (1 µl ml⁻¹) and limonene (0.2 µl ml⁻¹) dissolved in normal saline solution. All three chemicals were obtained from Sigma. It was ejected by pressure from a glass pipette (0.5-µm opening) with its tip brought within 20 µm from the cilia of the recorded cell. All experiments were at room temperature.

total of four experiments of the type shown in Fig. 3c and d, the Cl⁻ component was estimated to account for 43 ± 24% (mean ± s.d.) in peak current amplitude and 64 ± 21% in total charge influx during the overall olfactory response. As the reversal potentials for the cationic and anionic currents are comparable under these experimental conditions, the first percentage also indicates the relative contributions from the cation and anion conductances.

The foregoing results were obtained with whole-cell recordings using pipettes containing 120 mM Cl⁻. Thus it was possible that the inward Cl⁻ current might be caused by an artificially high [Cl⁻]_i due to exchange with the pipette solution. This seems unlikely, because the odorant-induced current in normal saline already appeared monophasic and had a reversal potential near 0 mV when examined immediately (within 10 s) after the establishment of whole-cell recording. To eliminate this possibility, however, we also performed perforated-patch recording with a nystatin-containing pipette solution³¹, which considerably slowed Cl⁻ exchange between the cell cytoplasm and the pipette interior³². Whether the pipette contained 120 or 0 mM Cl⁻, we found that the odorant-activated current initially still had a reversal potential near 0 mV. Furthermore, as shown in Fig. 4, even with a pipette containing no Cl⁻, there was still a SITS-sensitive component in the inward transduction current (measured at -65 mV). Thus, an inward Cl⁻ current in the olfactory response appears to be a physiological phenomenon. Put another way, the normal chloride concentration within the olfactory cilia must already be very high, probably as a result of a chloride uptake mechanism like that existing in certain types of neurons³³. In Fig. 4b, the Cl⁻ current is seen to follow the cationic current closely; thus, even a weak odorant stimulus eliciting a cationic current as small as a few pA is expected to

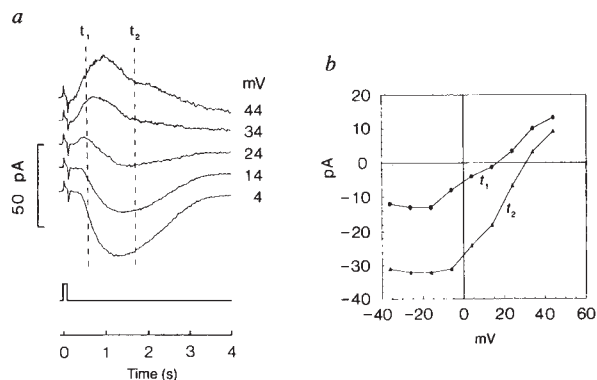
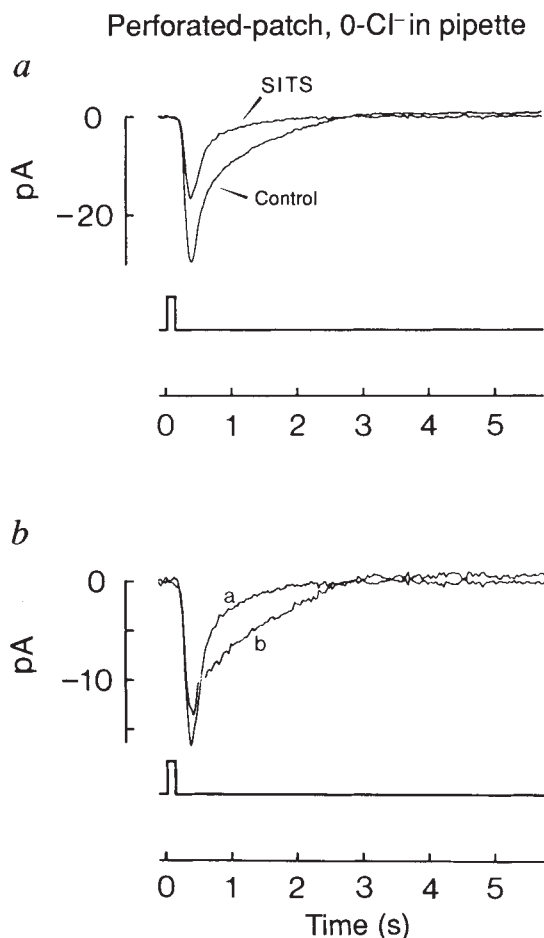
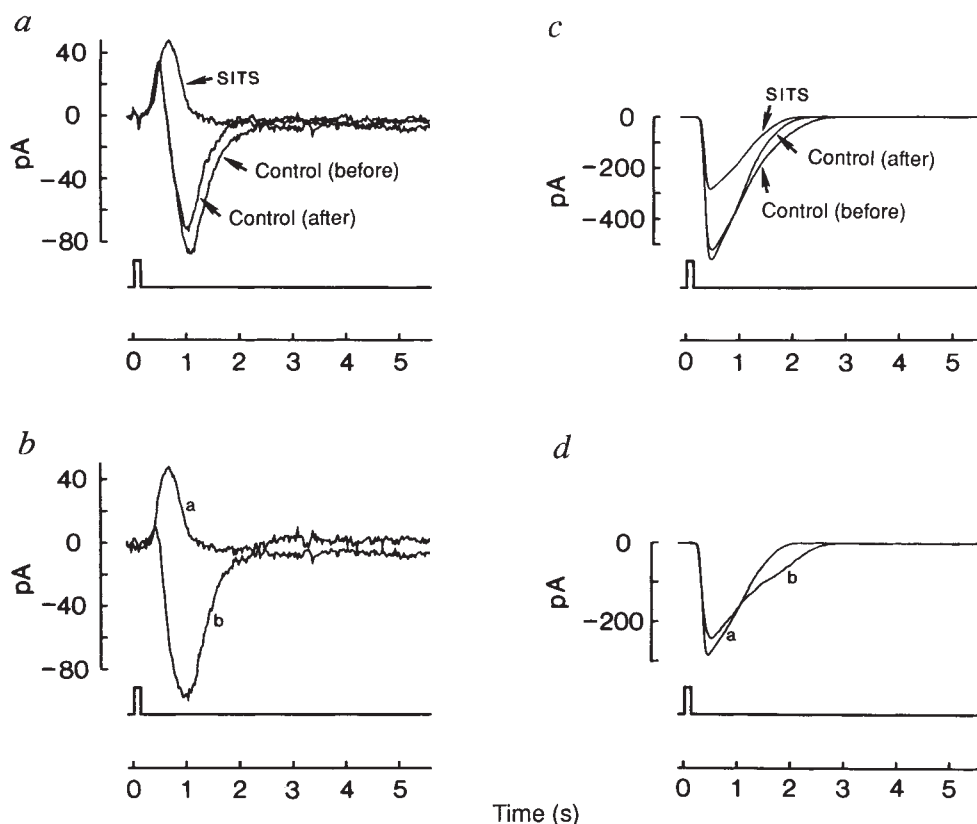


FIG. 2. *a*, Odorant-induced currents recorded in a low-Cl⁻ external solution. Newt receptor cell. The bath solution contained (in mM): 90 Na-gluconate, 30 NaCl, 2 CaCl₂, 10 Na-HEPES, 10 glucose, pH 7.4. Same whole-cell pipette solution as in Fig. 1. Each trace represents the response to a single stimulus trial. Note the biphasic waveform of the current response at +24 mV. Dashed lines indicate time instants (0.56 s and 1.68 s after the onset of the odorant stimulus) adopted for measurements that were used to construct the current-voltage relations shown in *b*. *b*, Current-voltage relations obtained from the experiment in *a*, including data at other potentials not shown in *a*.

lead to a Cl⁻ current. From seven such perforated-patch experiments (4 from newt and 3 from salamander), the Cl⁻ component accounts for 36 ± 25% in peak current amplitude and 60 ± 22% in total charge influx of the olfactory response. These estimates are broadly similar to those given earlier from whole-cell recordings using pipettes containing 120 mM Cl⁻, even though the two

FIG. 3. *a*, Effect of 2 mM external SITS (4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulphonic acid) on the odorant-induced biphasic response in low- Cl^- external solution. Newt receptor cell. Membrane potential at +34 mV. The bath solution contained (in mM): 120 Na-glucuronate, 2 CaCl_2 , 10 Na-HEPES, 10 glucose. Pipette solution contained 120 mM CsCl_2 , 2 mM Na-HEPES. The selective removal of the delayed inward current component was confirmed in four other cells (three from newt and one from salamander). *b*, Cationic and Cl^- components of the odorant-induced current in *a* plotted separately. Trace *a*, replot of (cationic) current isolated by the presence of 2 mM SITS as shown in *a*. Trace *b*, SITS-sensitive Cl^- current obtained as the difference between the current traces obtained in the presence and absence (before application) of SITS. *c*, Effect of 2 mM SITS on the odorant-induced current in physiological saline. Membrane potential was held at -54 mV. Same pipette solution as in *a*. Newt receptor cell. *d*, Comparison between cationic and Cl^- current components in the experiment of *c*. Trace *a*, cationic current; trace *b*, Cl^- current. In this experiment, the recordings were obtained starting ~2 min after the establishment of whole-cell recording.



experimental conditions represent opposite extremes. These numbers should therefore reasonably apply to the intact cell. The Cl^- contribution is very substantial, particularly for the total charge influx, which is the more important parameter to determine excitation of a receptor cell when the odorant stimulus lasts for more than a couple of seconds.

The olfactory receptor cells are unusual among sensory neurons in that their transduction machinery is in direct contact with the external environment. Although usually bathed in secreted mucus, the olfactory cilia may not always be exposed to constant ionic concentrations. A mucus dilution by, say, fresh water would reduce the transduction current if cations were the sole current carriers. When an accompanying Cl^- component is present, however, such a reduction in inward cationic current would be compensated by an increase in the inward anionic current, thus maintaining the effectiveness of the transduction mechanism. Earlier work²⁶ has shown that the electro-olfac-

FIG. 4 *a*, Same kind of experiment as in Fig. 3*c*, except perforated-patch recording was used with a pipette containing no Cl^- in order to avoid loading the cell with Cl^- . Membrane potential at -65 mV (holding potential was -50 mV, and liquid junction potential at the pipette tip was -15 mV). *b*, Separation of the odorant-induced inward current into cationic (trace *a*) and Cl^- (trace *b*) components as in Fig. 3*d*. Newt receptor cell. In this experiment, the recordings were obtained beginning about 2 min after establishment of gigaseal.

METHODS. For perforated-patch recordings, the pipette solution contained 120 mM K-glucuronate, 10 mM Na-HEPES, 5 mM Na-EGTA and 200 $\mu\text{g ml}^{-1}$ nystatin (diluted from a stock solution of 50 mg ml^{-1} in dimethylsulphoxide). After formation of the gigaseal, the conductance of the membrane patch gradually increased as indicated by the development of an outward potassium current at 0 mV, reflecting the incorporation of the ionophore into the membrane. Odorant stimulation was applied after the outward membrane current became stable, typically ~30 s after establishment of the gigaseal.

togram is, surprisingly, not diminished when all external NaCl is replaced with sucrose, and a similar finding has been made in unitary recordings from olfactory cells²⁷. Our experiments therefore provide a simple explanation for this observation. It will be interesting to see if a similar mechanism exists in mammalian olfactory receptor neurons. □

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Spatial calcium buffering in saccular hair cells

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THE potential importance of intracellular calcium-binding proteins in rapid and highly localized Ca^{2+} signalling is poorly understood. During fast synaptic transmission, which occurs at specialized active zones where Ca^{2+} diffuses only a few tens of nanometres from channels to neurotransmitter release sites¹, a cytoplasmic Ca^{2+} buffer would have to be extremely fast or present in millimolar concentrations to intercept a significant fraction of the calcium ions en route to their targets^{2–5}. Therefore, Ca^{2+} buffers have been presumed to be unimportant in fast exocytosis^{1,6,7} and another fast calcium-mediated process, electrical resonance in hair cells^{4,8}. Here I present evidence to the contrary by showing that hair cells in the frog sacculus contain millimolar concentrations of a mobile cytoplasmic calcium buffer that captures Ca^{2+} within a few microseconds after it enters through presynaptic Ca^{2+} channels and carries it away from the point of entry. This spatial buffering reduces the presynaptic free Ca^{2+} by up to 60 per cent and probably restricts the region in which the internal calcium ion concentration exceeds 1 μM to within <250 nm of each synaptic site. The buffer can thus influence both electrical resonance and synaptic transmission. Calbindin- $\text{D}_{28\text{k}}$ or a related protein may serve as the mobile calcium buffer, an action similar to its function in transporting Ca^{2+} across intestinal epithelial cells.

Frequency tuning of frog saccular hair cells by electrical resonance involves calcium channels and calcium activated potassium (K_{Ca}) channels^{9–11}, which occur in discrete clusters of ~40 K_{Ca} channels and ~90 calcium channels that appear to coincide with active zones^{4,8,12,13}. Here I infer the presynaptic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ps}}$) from the whole-cell K_{Ca} current, assuming this presynaptic localization of K_{Ca} channels. Exogenous calcium buffers were introduced through the recording pipette; the cell's native buffer properties were studied using perforated-patch recordings¹⁴.

Increasing the intracellular concentration of a fast Ca^{2+} buffer (BAPTA; $k_{\text{on}} = 6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (refs 15, 16) diminished the K_{Ca} current (Fig. 1a). A slower buffer (EGTA; $k_{\text{on}} = 1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (ref. 16) with similar equilibrium affinity for Ca^{2+} ($K_{\text{d}} \approx 100 \text{ nM}$) and a similar diffusional mobility was ineffective, suggesting that k_{on} is more important than K_{d} in determining a buffer's ability to reduce $[\text{Ca}^{2+}]_{\text{ps}}$. This conclusion is supported

by the equal effectiveness of BAPTA and Br_2BAPTA ($K_{\text{d}} = 5 \mu\text{M}$ (ref. 16), Fig. 3b), which have similar k_{on} values but different K_{d} values.

The small K_{Ca} current at –40 mV in perforated-patch recordings (Fig. 1a, thick trace) suggests that some cytoplasmic factor that normally shifts the K_{Ca} channels' voltage dependence to the right (Fig. 2) was lost in whole-cell recordings, presumably by diffusion into the recording pipette. This loss could be offset by adding 1.6 mM BAPTA to the pipette (Fig. 1a). This unknown factor does not directly alter the K_{Ca} channels' Ca^{2+} and voltage sensitivity (Fig. 2, filled circle), and therefore must operate by reducing $[\text{Ca}^{2+}]_{\text{ps}}$. I propose that it is a native cytoplasmic calcium buffer that captures calcium ions as quickly as 1–2 mM BAPTA.

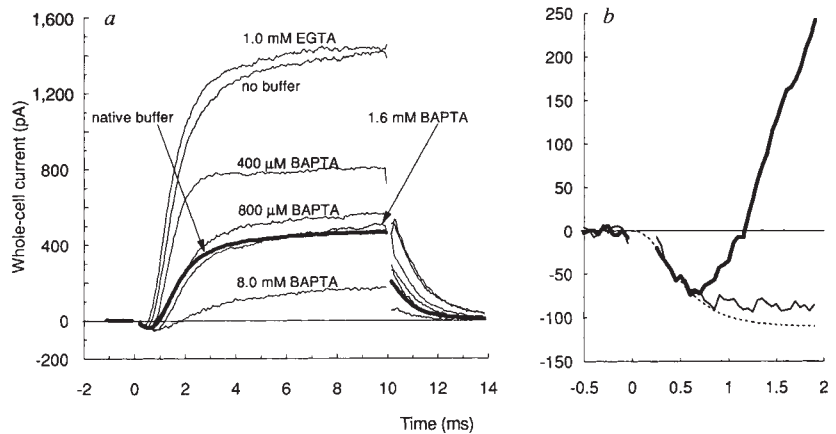
In Fig. 3a, the shallow slopes of the curves for 800 μM , 1.6 mM and 8 mM BAPTA show them to be effective buffers even when exposed to $\gg 1 \mu\text{M}$ $[\text{Ca}^{2+}]_{\text{ps}}$, which would saturate BAPTA at equilibrium. This is possible as long as calcium-free BAPTA is rapidly replenished by diffusion from the surrounding volume of cytoplasm. However, some local depletion must occur. Because BAPTA is unlikely to diffuse as fast as free Ca^{2+} , the reduction in $[\text{Ca-free BAPTA}]_{\text{ps}}$ must exceed the reduction in $[\text{Ca}^{2+}]_{\text{ps}}$ that it produces³. Thus, for 800 μM BAPTA to have reduced $[\text{Ca}^{2+}]_{\text{ps}}$ by 400 μM (from 1 mM to 600 μM) during a local Ca^{2+} influx of 20 ions per μs (Fig. 3a), the $[\text{Ca-free BAPTA}]_{\text{ps}}$ must have been reduced by >400 μM (>50%). Such local depletion causes an upward bending of the curve for 800 μM BAPTA during Ca^{2+} influxes >10 ions per μs (Fig. 3a). The native buffer was equivalent to ~500 μM BAPTA during the smallest Ca^{2+} fluxes (Fig. 3b), but was more resistant to depletion than 800 μM BAPTA during large fluxes (Fig. 3a), from which I conclude that the native buffer comprised >1 mM Ca^{2+} -binding sites.

These results support theoretical arguments^{2,3,5,17} against using equilibrium equations^{16,18–20} to model buffered diffusion over distances of a few micrometres or less. Under these conditions (mobile buffer, $[\text{Ca}^{2+}]_{\text{ps}} \gg K_{\text{d}}$), the steady-state Ca^{2+} concentration, $c(r)$, at a distance r from a point of steady Ca^{2+} influx is approximately³

$$c(r) = c_{\infty} + \frac{J_{\text{Ca}}}{2\pi r D_{\text{Ca}}} \exp(-r/\lambda),$$

where $\lambda = (D_{\text{Ca}}\tau_c)^{1/2}$ is a space constant, $\tau_c = 1/(k_{\text{on}}B)$ is the mean capture-time of the buffer, D_{Ca} is the Ca^{2+} diffusion coefficient ($\sim 3 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$), J_{Ca} is the local Ca^{2+} influx (mol s^{-1}), B is the buffer concentration, c_{∞} is the baseline $[\text{Ca}^{2+}]_i$ throughout the cell, and $K_{\text{d}} \gg c_{\infty}$. Because this equation ignores local depletion³, it is valid only for small Ca^{2+} fluxes (Fig. 3b).

FIG. 1 Whole-cell currents during voltage clamp steps from -70 mV to -40 mV ($t=0$) and back ($t=10$ ms). *a*, In each trace the current at -40 mV was initially negative (inward), owing to the fast opening of voltage-gated Ca^{2+} channels, but quickly reversed sign and approached a positive steady-state level representing the sum of the Ca^{2+} current and the larger, outward K_{Ca} current. Each trace is from a different cell and has been leak-subtracted and zeroed⁴. Traces were selected to have the same size Ca^{2+} current at -40 mV (that is the initial negative currents superimpose). The thick trace (native buffer) was from a perforated-patch recording, the rest were whole-cell recordings with Ca^{2+} buffers added to the pipette as labelled. When E_m was returned to -70 mV, the Ca^{2+} channels closed rapidly, while the K_{Ca} channels closed more slowly, producing exponentially decaying tail currents. The K_{Ca} channels' steady-state level of activation was determined by extrapolating these tail currents back to the instant at which E_m was returned to -70 mV (see Fig. 3). *b*, Verification that the Ca^{2+} current amplitude (I_{Ca}) can be estimated by extrapolating the initial negative current. Whole-cell currents recorded through a perforated patch before (*thick trace*) and 30 s after (*thin trace*) blocking K_{Ca} channels with $1 \mu\text{M}$ charybdotoxin (Peptides International, Louisville, KY) to uncover the Ca^{2+} current. The dashed trace was calculated from ref. 11 as $I_{\text{Ca}} = I_{\text{Ca}}(1 - \exp(-t/\tau))^3$, where I_{Ca} was adjusted to give the best fit to the initial negative current (*thick trace*). I_{Ca} is a reasonable approximation to the steady-state I_{Ca} (*thin trace*). In this and all fits used for Fig. 3, $\tau = 300 \mu\text{s}$. METHODS. Recordings were from enzymatically dissociated frog saccular hair cells (*Rana pipiens*) using glass pipettes sealed tightly to the cell surface. Access to the cell interior was achieved by rupturing the membrane inside the pipette (whole-cell recording), or through ion channels formed by nystatin in the pipette¹⁴ (perforated-patch recording). In whole-cell recordings, it took ~ 60 s for the soluble components of the cytoplasm to exchange with the pipette solution through its $2\text{--}4 \mu\text{m}$ -diameter tip²⁸. Extracellular



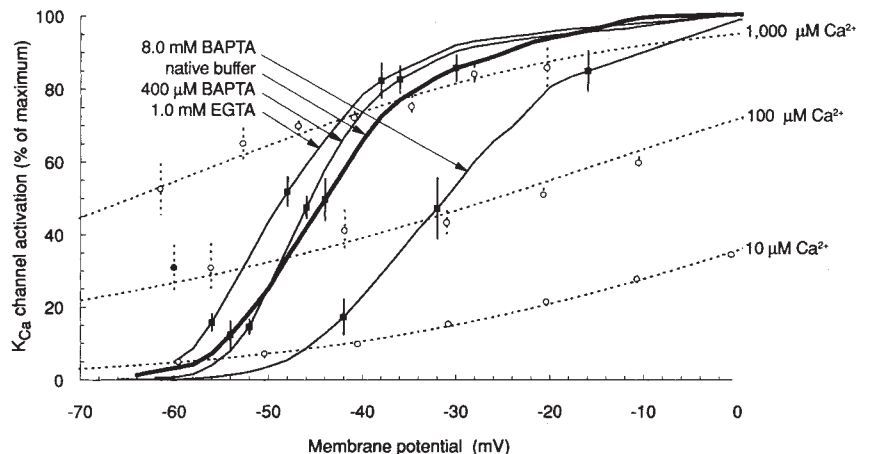
saline composition (in mM): 110 Na^+ , 2 K^+ , 4 Ca^{2+} , 118 Cl^- , 5 HEPES, 3 D-glucose, pH 7.25. Pipette solution: 116 K^+ , 106 aspartate, 2 Mg^{2+} , 5 HEPES, 1 ATP, pH 7.25. Ca^{2+} buffers: 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA; Molecular Probes), 5,5'-dibromo-BAPTA (Br_2BAPTA ; Molecular Probes), and ethylene-glycol-bis-(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; Sigma). Perforated-patch pipettes were filled with the same pipette solution, with added EGTA (1 mM), Ca^{2+} (78 μM), and nystatin (100-fold dilution from a stock solution of 50 mg nystatin per ml dimethylsulphoxide). Access resistances were 2–4 M Ω in whole-cell and 3–6 M Ω in perforated-patch recordings, 80% compensated. The zero-current potential was set to -13 mV (the diffusion potential between the pipette solution and extracellular saline¹⁰) before contacting the cell, and checked for drift after each recording. Recordings were rejected if the drift was >4 mV. Consistent effects of BAPTA were found only in cells with steady 'leak' currents <50 pA at -70 mV; about half of the cells met this criterion and the rest were discarded. Cells were also rejected if the maximum K_{Ca} tail current amplitude was <500 pA. All whole-cell recordings were made at least 90 s after breaking onto the cell.

The values of λ and τ_c for the native buffer must therefore approximate the values calculated for 500 μM BAPTA ($\lambda = 32$ nm, $\tau_c = 3.4 \mu\text{s}$). This λ is small enough to account for the observed $\sim 60\%$ reduction in $[\text{Ca}^{2+}]_{\text{ps}}$, assuming a 300-nm-diameter channel cluster⁴. At a distance of 250 nm from the edge of the cluster ($r > 7\lambda$), the buffer is expected to attenuate $c(r)$ by a factor $>1,000$ (that is $>\exp(7)$); without the buffer, the $[\text{Ca}^{2+}]_i$ at this distance would still be $\sim 10\%$ of the value at the centre of the cluster⁸ (namely 1–100 μM). Thus, the mobile

buffer both reduces the local rise in $[\text{Ca}^{2+}]_i$ within a cluster of channels and restricts the region of elevated $[\text{Ca}^{2+}]_i$ to a domain of submicron dimensions. Unlike fixed buffers, which can only suppress transient changes in $[\text{Ca}^{2+}]_i$, the effect of a mobile buffer on a standing spatial Ca^{2+} gradient persists indefinitely and may thus provide prolonged protection from Ca^{2+} cytotoxicity²¹.

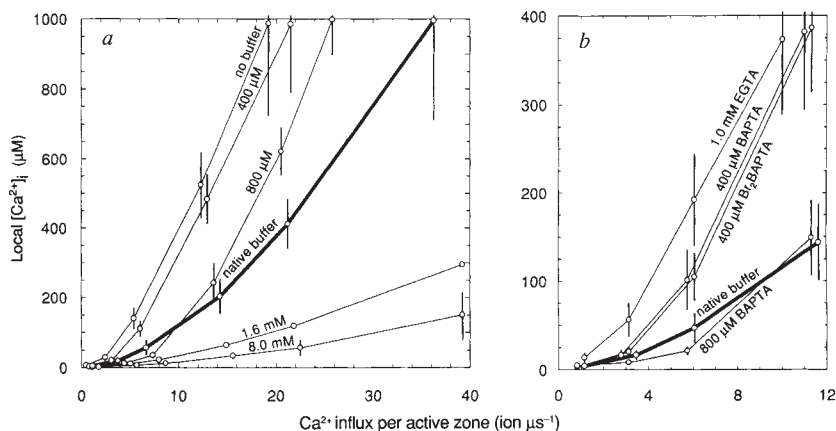
Calbindin (M_r 28K, 5–6 Ca^{2+} -binding sites²²) and calmodulin (M_r 20K, 4 Ca^{2+} -binding sites), or similar proteins, are abundant

FIG. 2 The effects of Ca^{2+} buffers on the apparent voltage dependence of the K_{Ca} current. Solid lines show steady-state K_{Ca} channel activation averaged from tail currents (Fig. 1) in 46 cells. Data were taken at voltage increments of 3–10 mV, normalized to the maximal K_{Ca} current in each cell, and interpolated linearly between measurements when averaging data from different cells. For clarity, s.e.m. values (bars) are shown at only a few voltages. To calibrate the K_{Ca} current's Ca- and voltage-dependence, cells were studied with the Ca^{2+} current blocked and highly buffered Ca^{2+} concentrations in the whole-cell pipette⁴. These data points ($\circ$) show the K_{Ca} channel's probability of being open as a function of E_m for $[\text{Ca}^{2+}]_i = 10, 100$ and 1,000 μM . Error bars (s.e.m.) are shown when they exceeded the size of the symbol. These calibration values were used to fit parameter values in a model¹⁰ that describes the K_{Ca} channels' Ca and voltage dependence. The model's predictions of K_{Ca} channel activation as a function of E_m and $[\text{Ca}^{2+}]_i$ (dashed curves) were found to fit to the constant $[\text{Ca}^{2+}]_i$ data well, and were used in Fig. 3 to calculate the $[\text{Ca}^{2+}]_{\text{ps}}$ that must have been present to obtain the observed K_{Ca} channel activation. One additional calibration point ($\bullet$) was obtained in perforated-patch recordings. These cells were loaded with Ca^{2+} by holding



them depolarized until the prolonged Ca^{2+} current overwhelmed the cell's capacity to sequester Ca^{2+} , as evidenced by a shift of the Ca^{2+} current's reversal potential to near the holding potential. A 30-s depolarization to $+19$ mV, with external $[\text{Ca}^{2+}] = 500 \mu\text{M}$, was sufficient to raise $[\text{Ca}^{2+}]_i$ to its equilibrium concentration of 100 μM . K_{Ca} current activation was then measured following repolarization to -60 mV.

FIG. 3 The relationship between local Ca^{2+} influx and accumulation. At each E_m and buffer concentration, the $[\text{Ca}^{2+}]_{\text{ps}}$ was determined from the percentage activation of K_{Ca} current using data and calibrations as for Fig. 2. The local Ca^{2+} influx was estimated by dividing the total Ca^{2+} current (determined separately at each E_m in each cell as shown in Fig. 1b) by 19 (the average number of presynaptic sites per cell). a, Large Ca^{2+} fluxes. The 'no buffer' curve includes data from experiments using 1 mM EGTA, which was found to have no effect. The '400 μM ' curve includes data using BAPTA and Br_2BAPTA . b, comparison of EGTA, BAPTA and Br_2BAPTA during small Ca^{2+} fluxes.



in frog saccular hair cells^{23,24} and may bind Ca^{2+} and diffuse quickly enough ($k_{\text{on}} > 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$; $D > 10^{-10} \text{ m}^2 \text{ s}^{-1}$) (refs 5, 19) to be important mobile Ca^{2+} carriers. Calbindin is an attractive candidate because related proteins (calbindin-9K and calbindin-28K) are mobile Ca^{2+} carriers in kidney¹⁸ and intestinal¹⁹ epithelial cells, and because it is found at the highest concentrations in cells that relay auditory phase information to the brain²⁵, suggesting a role in fast synaptic transmission. Some hair cells may contain $> 1 \text{ mM}$ calbindin²⁶ ($> 5 \text{ mM}$ Ca^{2+} -binding sites); certain neurons have high concentrations of calbindin as well²⁷. Whatever its identity, the mobile Ca^{2+} buffer is likely to have important consequences for electrical tuning and synaptic transmission in hair cells. □

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Calcitonin gene-related peptide potentiates synaptic responses at developing neuromuscular junction

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PROTEIN phosphorylation is important in synaptic transmission and plasticity¹. At the neuromuscular junction, phosphorylation of acetylcholine (ACh) receptor-channels increases the rate of agonist-induced channel desensitization². In contrast, potentiation of ACh channel activity through protein phosphorylation has not been described. We report here that calcitonin gene-related peptide (CGRP), a neuropeptide present at presynaptic motor nerve terminals^{3,4}, enhances the postsynaptic response at developing neuromuscular junctions by increasing the burst duration of embryonic ACh channels. The effect of CGRP on these ACh channels is mimicked by dibutyryl-cyclic AMP and by cAMP-

dependent protein kinase (PKA) and prevented by a specific peptide inhibitor of PKA. Moreover, postsynaptic inhibition of PKA reduced the amplitude and decay time of spontaneous synaptic currents, suggesting that endogenous CGRP may act as a potentiating factor during the early phase of synaptogenesis.

The effect of CGRP on spontaneous synaptic currents (SSCs) was examined in 1-day-old *Xenopus* nerve-muscle cultures^{5,6}. Amplitude and decay time, but not rise time, exhibited significant increases after CGRP treatment (Fig. 1; Table 1a). The effects of CGRP are readily seen in the histograms of SSC amplitudes (Fig. 1c) and decay times (Fig. 1d). The peptide had no effect on the frequency of SSCs, which was $119 \pm 21\%$ (mean $\pm$ s.e., $n = 6$) of control after CGRP treatment. Thus CGRP appears to regulate spontaneous synaptic activity through a postsynaptic mechanism, rather than by affecting presynaptic ACh release.

To characterize the postsynaptic effect of CGRP, single ACh channel activities were recorded on isolated myocytes⁷. Very low concentrations of ACh (0.2–100 pM) were used in the recording pipette to avoid agonist-induced channel desensitization. ACh activated ACh channels at these low concentrations: the frequency of single channel events increased with ACh concentration over this range (Fig. 2a), and the single channel currents were completely abolished by pretreatment of the cell with α -bungarotoxin ($5 \mu\text{g ml}^{-1}$).

Two populations of ACh channels have been observed^{8,9}: a low-conductance channel with long burst duration, which is the predominant population in 1-day-old cultures, and a high-conductance channel with short burst duration. Application of CGRP to 1-day-old cultures specifically increased the burst

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duration of the low-conductance channels (Fig. 2b, c, and Table 1b), without affecting the high-conductance channels. No significant change in the amplitudes of single channel currents was found for either channel type (Fig. 2b, c; Table 1b), indicating that channel conductances remained the same.

We also compared the effects of CGRP on myocytes at different developmental stages. The mean burst duration of low-conductance channels in myocytes decreased from 4.3 ms in 1-day-old cultures to 1.2 ms in 3-day-old cultures (Table 1b), with a concomitant decrease in the relative number of this type of channels⁹. CGRP treatment, which significantly increased the burst duration of the low-conductance channels in 1-day cultures, had no effect on myocytes in 3-day cultures (Table 1b). Thus, the CGRP effect is restricted to the early phase of synaptogenesis, when this type of channel plays a predominant role. The lengthening of channel burst duration by CGRP at early neuromuscular synapses, by increasing overlaps of ACh channel openings, can account for the increase in amplitude and decay time of spontaneous synaptic currents. The lack of CGRP effects in older myocytes is likely to result from changes in the properties of ACh channels, rather than an absence of CGRP receptors, because CGRP is still capable of regulating ACh channel synthesis^{10,11} and desensitization¹² later in development.

CGRP binds to specific receptors¹³⁻¹⁵, elevates cAMP levels¹⁶ and stimulates the phosphorylation of ACh channels in myocytes¹⁷. To examine whether CGRP might act through cAMP-dependent protein kinase (PKA), single ACh channel activities were recorded before and after loading the catalytic subunit of PKA into isolated myocytes. As shown in Fig. 3a, loading of PKA mimicked the action of CGRP in lengthening the burst duration of low-conductance channels. A similar effect was observed on bath application of dibutyryl-cAMP, a membrane-permeable analogue of cAMP. Moreover, the CGRP effect was abolished by pre-loading a specific PKA peptide inhibitor (PKI; 500 μ M) into the myocytes. The effect of PKI was much less effective when a 10-fold lower concentration (50 μ M) was

TABLE 1 Effect of CGRP on SSCs and ACh channel kinetics

(a) Spontaneous synaptic currents					
	Amplitude (pA)	Decay time (ms)	Rise time (ms)	No. of cells	
Control	305 ± 46	5.0 ± 0.5	0.68 ± 0.06	6	
CGRP	488 ± 54*	6.8 ± 0.5*	0.65 ± 0.08		
Ratio†	1.7 ± 0.1**	1.4 ± 0.1**	0.96 ± 0.07		
(b) Single ACh channel currents‡					
	Low conductance		High conductance		No. of cells
	τ_1 (ms)	I_1 (pA)	τ_2 (ms)	I_2 (pA)	
Control (1-day)	4.3 ± 0.3	4.2 ± 0.2	1.5 ± 0.1	6.1 ± 0.3	8
CGRP (1-day)	7.3 ± 0.5**	4.2 ± 0.2	1.8 ± 0.3	6.1 ± 0.2	
Control (3-day)	1.2 ± 0.1	4.5 ± 0.1	1.3 ± 0.1	6.2 ± 0.1	6
CGRP (3-day)	1.3 ± 0.1	4.4 ± 0.1	1.2 ± 0.1	6.3 ± 0.1	

Data were collected as paired measurements for the same cell over equal duration before and 2–5 min after CGRP treatment. Rise time refers to interval between 10% and 90% of peak SSC amplitude. All values represent mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.001$ (t -test).

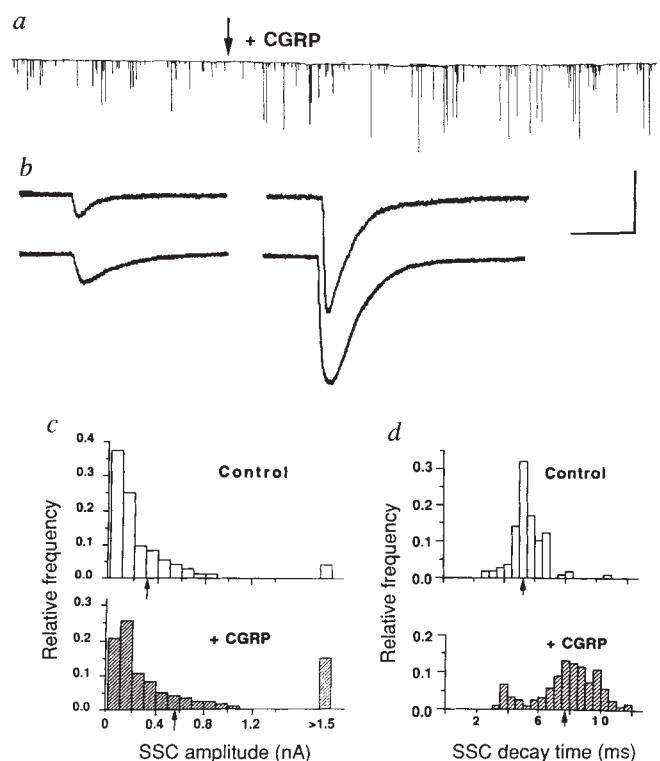
† The ratio between the mean value after CGRP treatment and the mean control value was calculated for each cell before averaging.

‡ τ , Mean burst duration; I , amplitude of single channel current.

used. It is unlikely that the regulation of channel burst duration was due to changes in membrane potential, because the amplitude of single ACh channel currents did not change after any of these treatments (data not shown). PKA-dependent phosphorylation increases the opening probability and open time of purified ACh channels reconstituted in lipid bilayers in the

FIG. 1. Effects of CGRP on spontaneous synaptic currents (SSCs) in 1-day-old *Xenopus* nerve-muscle cultures. *a*, Continuous trace depicting the membrane current recorded from an innervated myocyte before and after bath application of CGRP (final concentration, 0.7 μ M). Downward deflections are SSCs ($V_h = -70$ mV, filtered at 150 Hz). *b*, Samples of SSCs before (upper traces) and after (lower traces) CGRP treatment are shown at higher time resolution (filtered at 3 kHz). Calibration bars, 0.6 nA, 2 min for *a*; 0.25 nA, 20 ms for *b*. *c*, Amplitude distribution of SSCs plotted as relative frequency of events (fraction of total events) with different amplitudes. Data were collected and analysed from the same cell as that shown in *a*. All events within a period of 16 min before and 16 min after CGRP application were used in the analysis. Arrows indicate the mean values, which were 0.312 nA for the control and 0.516 nA after CGRP treatment. *d*, Decay time distribution of SSCs plotted as relative frequency of events (fraction of total events) with different decay times. Data were collected by the same procedure as in *c*. The decay phase of SSCs was fitted by a single exponential curve and decay time was defined as the time needed for SSC amplitude to drop to $1/e$ of the peak value. Arrows indicate the mean values, which were 5.2 ms for the control and 7.6 ms after CGRP treatment.

METHODS. *Xenopus* nerve-muscle cultures were prepared as previously described⁵. Briefly, the neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos (stage 20–22) were dissociated in Ca^{2+} - and Mg^{2+} -free saline supplemented with EDTA. The cells were plated on clean glass coverslips and were used for experiments after 24-h incubation at room temperature (20–22 °C). SSCs were recorded from innervated myocytes in the whole-cell configuration⁶ with voltage clamped at -70 mV. The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM CaCl_2 , 2.5 mM KCl, 10 mM HEPES, pH 7.6), 49% L-15 Leibovitz medium (Sigma), and 1% fetal bovine serum (GIBCO). The internal pipette solution contained 3 mM Mg-ATP, 150 mM KCl, 1 mM NaCl, 1 mM MgCl_2 and 10 mM HEPES (pH 7.2). CGRP was added to the culture medium in a final concentration of 0.5–1 μ M. All data were collected by a patch clamp amplifier (EPC-7), with a current signal filter at 3 kHz. The data were stored on a videotape recorder for later playback on a storage oscilloscope (Textonic 5113) and



a chart recorder (Gould RS3200). The amplitude and decay time of SSCs were analysed by EGAA program (RC Technology).

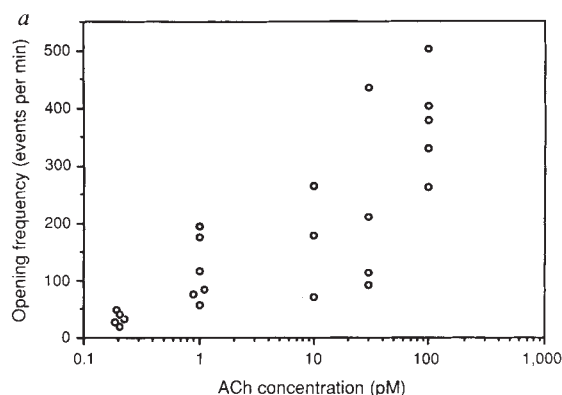
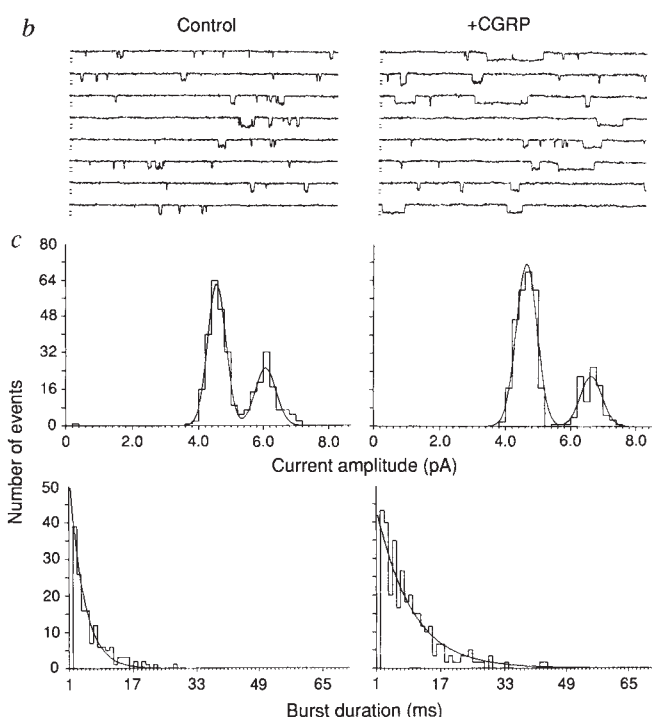


FIG. 2 *a*, Activation of ACh channel at low concentrations of ACh. The opening frequency of the ACh channel (defined as number of opening events per min) was plotted against ACh concentration in the patch pipette. Each point represents the opening frequency recorded from a single myocyte at a given ACh concentration. Repeated patch recordings were made on the same myocyte with pipettes containing different concentrations of ACh. Data from 7 cells in 6 cultures are presented. *b, c*, Effects of CGRP on single ACh channel currents. *b*, Samples of single channel currents recorded from the same myocyte before and 18 min after CGRP application (0.7 μ M), using two consecutive recording pipettes filled with 0.2 pM ACh. A single sweep represents 102.4 ms. Approximate current levels of low (4 pA) and high (6 pA) conductance channels are indicated at the beginning of each sweep. *c*, Upper panels: amplitude histograms of single ACh channel currents recorded from the same cell as that shown in *b*. Total numbers of current events were 332 and 363, respectively, before and after CGRP treatment. Each amplitude histogram was fitted with two gaussian distribution curves (solid lines), and the calculated values of the mean current amplitude (in pA) are: control, I_1 (low-conductance) = 4.56 and I_2 (high-conductance) = 6.07; after CGRP, I_1 = 4.64 and I_2 = 6.63. *c*, Lower panels: distribution of burst durations of low-conductance channels for the same data as in upper panels. Solid lines represent the best-fits with single exponential curves. The burst duration distribution for the high-conductance channels is not shown. The calculated mean values (in ms) are: control, τ_1 (low-conductance channel) = 3.54 and τ_2 (high-conductance channel) = 1.35; after CGRP, τ_1 = 8.78 and τ_2 = 1.52.

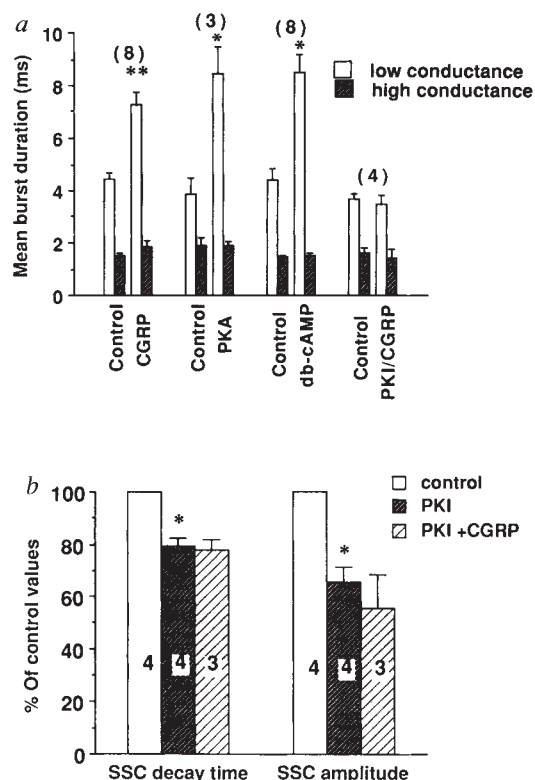
METHODS. *a*, Cell-attached patch recordings were made on isolated myocytes in a 1-day-old *Xenopus* culture, at an applied pipette potential of 60 mV.



The pipette, which had been coated with Sylgard (Dow Chemical) before polishing, contained normal Ringer's solution plus the indicated concentration of ACh. Membrane currents were recorded by a patch clamp amplifier (List, EPC-7) and stored on a videotape recorder. The data were filtered at 3 kHz, digitized at 50 μ s intervals and analysed with pClamp program (Axon Instruments). Single channel analysis was done essentially as described.⁷ The low- and high-conductance channel currents, which were about 4 pA and 6 pA, respectively, under 60 mV pipette potential, were determined by the program with a maximum likelihood method. Events corresponding to simultaneous openings of more than one channel, which were rare at the low ACh concentrations used in these experiments, were excluded from analysis. Events briefer than 200 μ s were not analysed because they were distorted by the limited frequency response of the instrument.

FIG. 3 *a*, Involvement of cAMP-dependent protein kinase (PKA) in the effect of CGRP on ACh channels. The burst duration of low- and high-conductance ACh channels was compared, by recordings with two consecutive cell-attached patch pipettes on the same cell, before and after various treatments. db-cAMP, Dibutyryl cAMP; PKI, Walsh peptide inhibitor. PKI/CGRP refers to treatment first with PKI and then with CGRP. *b*, Effects of PKI on properties of SSCs. After a 20-min period of recording (control), PKI (500 μ M) was loaded into the innervated myocyte with a second whole-cell pipette, which was used to record SSCs in the presence of the inhibitor for a second 20 min period (PKI). In 3 out of 4 experiments, CGRP was then added at a final bath concentration of 1 μ M, and recording continued for a further 20 min period (PKI + CGRP). The differences between control values and those after PKI treatment were statistically significant (both *t*-test and nonparametric tests, * $P < 0.05$). CGRP produced no significant change in either decay time or amplitude of SSCs after PKI treatment ($P > 0.5$).

METHODS. CGRP and db-cAMP were added to the bath at final concentrations of 0.7 μ M and 1 mM, respectively. Between two sets of single channel recordings, purified PKA catalytic subunit (0.5 mg ml⁻¹, dialysed in internal pipette solution) or PKI (500 μ M, dissolved in internal pipette solution) was loaded into the myocyte through a whole-cell pipette. The proteins were allowed to diffuse into the cell for 5–7 min and the pipette was then gently removed from the cell. CGRP was added to the bath after removal of the pipette containing PKI. Data are presented as mean $\pm$ s.e.m. The number of cells examined is indicated in the histogram. The difference between control values and values after various treatments was analysed by *t*-test and nonparametric (Mann-Whitney) tests, and the results from both tests were in agreement (* $P < 0.05$; ** $P < 0.01$).



absence of agonists¹⁸. Taken together, these results suggest that the CGRP-induced potentiation of the ACh response is mediated by PKA.

If endogenous CGRP released at the developing synapse plays a role in regulating the synaptic response, one would predict that postsynaptic inhibition of PKA might alter the synaptic currents at the early synapse. We found in each of four experiments that loading PK1 into innervated myocytes resulted in significant reduction of both SSC amplitude and decay time (Fig. 3b). Exposure to exogenous CGRP after loading PK1 into the myocytes produced no increase in the synaptic response. Thus endogenous CGRP may be functioning at the developing synapse in 1-day culture. We cannot exclude the possibility that the PK1 effect is due to antagonism of a neuromodulator other than CGRP.

In previous studies, CGRP¹³, PKA¹⁹, protein kinase C²⁰ and protein tyrosine kinase²¹ increased the rate of agonist-induced desensitization of ACh channels. Relatively high ACh concentrations were used in those studies, which may have prevented observation of a potentiating effect on the ACh channel. Alternatively, the enhancing effect of CGRP may occur only during an early phase of synaptic development: we observed no effect of CGRP on ACh channel burst duration in myocytes of older cultures (Table 1b).

Modulation of ACh channels by CGRP-regulated protein phosphorylation may be important for synapse development. At developing neuromuscular junctions, spontaneous synaptic activity appears immediately after nerve-muscle contact²². This activity plays a pivotal role in the maturation of synaptic connections, as well as in the differentiation of contractile properties of the muscle cells^{23–25}. CGRP is expressed concomitantly with the development of the neuromuscular junction²⁶. In motor neurons, CGRP is released in a Ca²⁺-dependent manner²⁷. We have now shown that CGRP enhances synaptic responses by increasing the burst duration of ACh channels. An increase in ACh channel burst duration was also observed when *Xenopus* muscle cells were co-cultured with neural tube tissue or treated with neural tube-conditioned medium²⁸, which may contain CGRP-like factors. Lengthening the burst duration of existing channels provides a means of elevating postsynaptic activity early in development, when time resolution of the synaptic event is less critical. □

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Repression of *c-fos* promoter by MyoD on muscle cell differentiation

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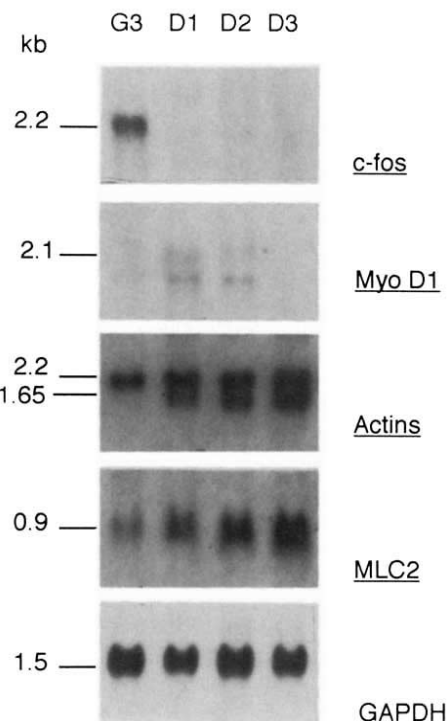
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TERMINAL differentiation and cell proliferation are in many cases, as in muscle cells¹, mutually exclusive processes. While differentiating myoblasts are withdrawn from the cell cycle², myogenesis is inhibited by some mitogens and overexpression of some oncogenes^{3,4}, including proto-oncogene *c-fos*⁵ (which expresses a growth-associated protein constituting the regulatory factor AP-1 in conjunction with *c-Jun*^{6,7}). MyoD, a muscle-specific transcription factor of the basic helix-loop-helix family^{8,9}, acts at both levels because it triggers a muscle differentiation programme in non-muscle cells^{10–12}, and induces a complete block of cell proliferation^{13,14}. Antagonistic interaction between MyoD and *c-Jun* has been demonstrated¹⁵. We here show that *c-fos* expression greatly decreases upon muscle cell differentiation, concomitant with MyoD-induced activity. We have identified a MyoD-binding site overlapping with the serum-responsive element in the *c-fos* promoter. We demonstrate that MyoD can act as a negative regulator for *c-fos* transcription by blocking serum responsiveness through

FIG. 1 *c-fos* mRNA is greatly decreased at the onset of muscle-cell terminal differentiation. Cells from a subclone of the rat myoblastic cell line L6 (L6- α 1²⁵, in which MyoD mRNA is inducible) were grown to confluency (G3) and induced to differentiate for one (D1), two (D2) or three (D3) days. Total RNA was isolated and analysed by northern blotting using probes for *c-fos*, MyoD1, muscle differentiation marker genes (actins, 1.65 kilobases (kb): α -actin; MLC2: myosin light chain 2) or an internal control (GAPDH, glyceraldehyde-3-phosphate dehydrogenase), as indicated. Size of the transcript is indicated on the left (in kb).



METHODS. L6 α 1 (a subclone of the rat L6 myoblastic cell line²⁵) were grown in DMEM supplemented with antibiotics and 10% fetal calf serum (FCS). Differentiation was induced by switching the culture medium to differentiation medium (2% FCS). Total RNA was extracted at the indicated time and 25 μ g were analysed by northern blotting using standard procedures. Probes (described in ref. 25) were, respectively, a 1.1 kb *Pst*I insert from *v-fos*, a 1.8 kb *Eco*RI insert from pMSV MyoD, a 1.2 kb *Pst*I insert from pMA91, a 0.5 kb *Pst*I insert from pMLC2-18 and a 1.2 kb *Pst*I insert from pRGADPH13.

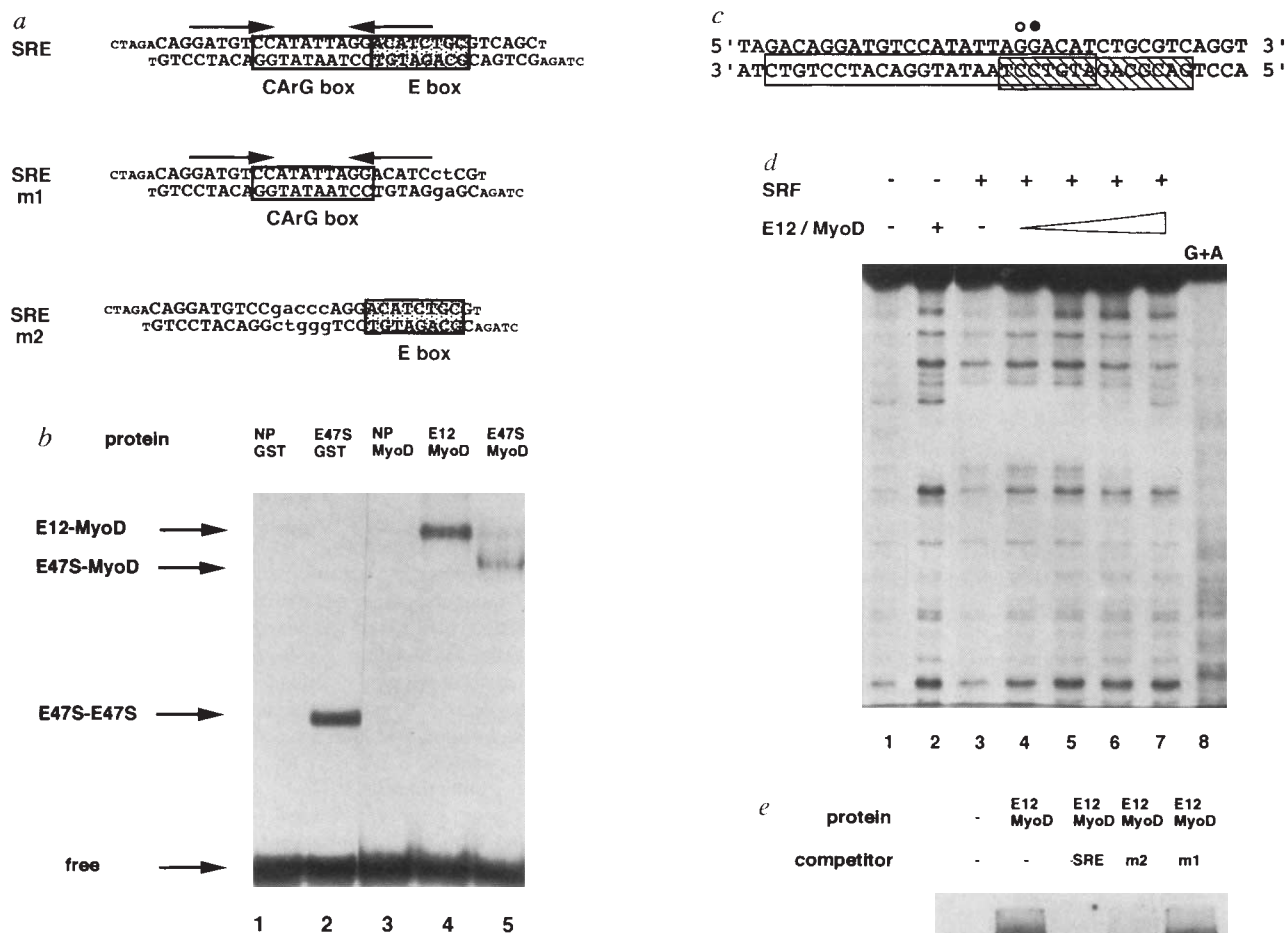


FIG. 2 MyoD recognizes an E-box in the SRE. **a**, Nucleotide sequence of the wild-type (SRE) and mutant (SREm1 and SREm2) oligonucleotides used in this study; shaded box indicates an E-box, open box indicates the CArG box and arrows delineate the dyad symmetry element. Lower-case letters indicate mutations and small capitals a restriction site. **b**, Electrophoretic mobility shift assay (EMSA) analysis of basic helix-loop-helix (preformed homo- or heterodimers) complexes²⁶. Arrows, position of protein complexes and free probe (wild-type SRE oligonucleotide). **c**, Binding site for MyoD/E12, assessed by DNase I footprinting (solid box) and methylation interference (solid circle, strong interference; open circle, partial interference). Open box, SRF protection. **d**, Binding of SRF and MyoD/E12 is mutually exclusive. A combination of bacterial SRF (0.1–0.2 μ g) and bacterial E12/MyoD (lane 4, ~0.07 μ g; lane 5, ~0.25 μ g; lane 6, ~0.7 μ g; lanes 2 and 7, ~1 μ g) was used for footprinting; lane 1, no protein; lane 8, chemical sequence. **e**, Binding of E12/MyoD is sequence-specific. E12/MyoD heterodimers were analysed as in **b**, with a 50-fold molar excess of indicated unlabelled competitors. METHODS. pE12 and pE47S are described in ref. 8 and pGEX-MyoD in ref. 9. pGEX-SRF has been constructed by inserting a *Bgl*III insert from pG3.5 vector²⁰ into the *Sma*I site of pGEX vector (Pharmacia) followed by mung bean exonuclease treatment. pGEX-E12 was constructed by inserting a *Sac*I-*Eco*RI fragment from pE12⁸ into the *Sma*I site of the pGEX vector.

this binding site. These data suggest that the MyoD negative effect on cell growth could be partly mediated by transcriptional inactivation of growth-responsive genes.

By northern analysis of differentiating L6 myoblastic cells (Fig. 1) we showed that, preceding the peak of transcription of muscle genes (α -actin and myosin light chain 2, MLC2: Fig. 1), *c-fos* expression decreased greatly, concomitant with the increase of MyoD messenger RNA expression (Fig. 1). We reasoned that MyoD could act as a downregulator of *c-fos* transcription. Indeed, a consensus MyoD-binding site, CANNTG¹⁶, is located within the serum-responsive element (SRE) in the *c-fos* promoter¹⁷ (E-box in Fig. 2a). The SRE is responsible for *c-fos* transcriptional activation by a variety of

pE12 and pE47S were *in vitro* translated according to ref. 8. GST-MyoD, GST-E12 and GST-SRF were purified according to ref. 9. Heterodimers were performed (15 min, 37 °C). The probe for footprinting analysis (³²P-end-labelled on the reverse strand) was derived by polymerase chain reaction (PCR).

growth-inducing signals^{18,19} and binds, in nonmuscle cells, a complex of proteins including serum response factor (SRF) and p62^{1CF} (refs 20, 21).

DNA-binding analysis (Fig. 2b) demonstrated that, as for muscle gene enhancer E-boxes²², MyoD needs to heterodimerize with either E12 (lane 4) or E47 (lane 5) to recognize this element. Furthermore, as usually observed¹⁶, the SRE was efficiently recognized by E47/E47 homodimers (lane 2) and not E12/E12 homodimers (not shown). DNase I footprinting and methylation interference experiments (Fig. 2c) indicated that the binding sites of SRF and E12/MyoD overlapped significantly. Additional experiments, using both proteins in various combinations, suggested that binding was mutually exclusive: the footprint

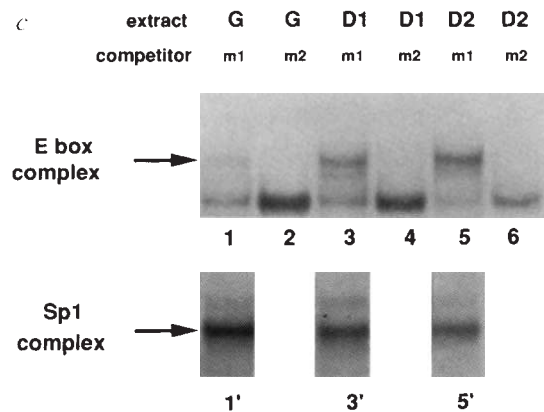
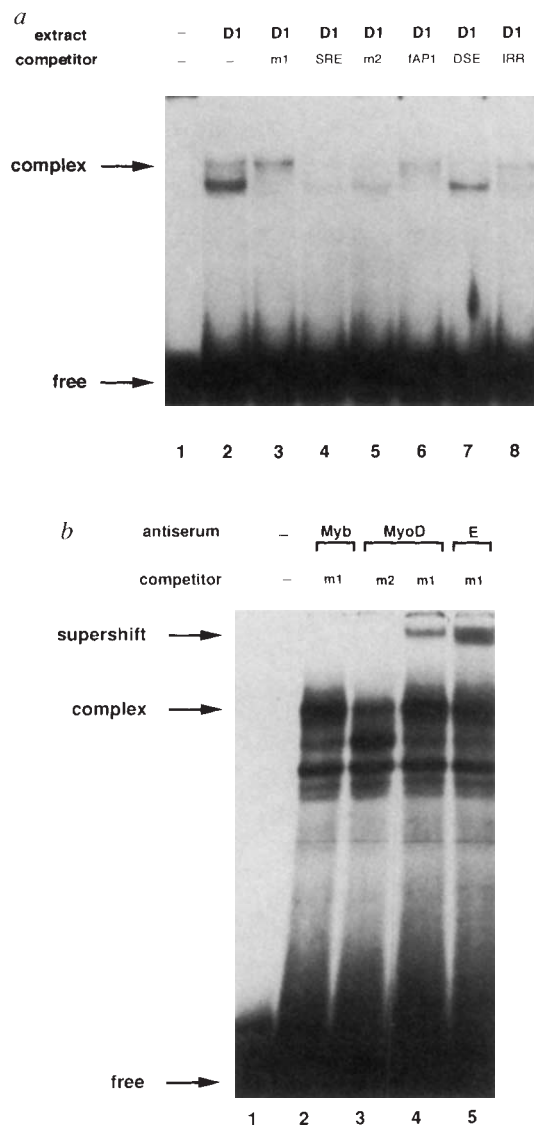


FIG. 3 A myoblastic nuclear protein of the MyoD family specifically recognizes the E-box in the SRE. **a**, EMSA analysis of binding specificity. Nuclear extracts from myoblasts at day 1 of differentiation (D1) were analysed by EMSA using the SREm2 oligonucleotide as a probe. A 50-fold molar excess of unlabelled competitor was added: fAP1: 5'-ATCTGCGTCAGCAGG-3'; DSE: 5'-CAGGATGTCCATATTAGGACATCTG-3'; IRR (irrelevant oligonucleotide) 5'-TTCCCCATGGTTTCCCTGTAGC-3'. Arrows denote the specific complex and the free probe. **b**, Proteins of the complex are antigenically related to MyoD and E12/E47. EMSA analysis of differentiated myoblasts (probe: SREm2) in the presence of an anti-Myb (Myb), an anti-MyoD (MyoD) or an anti E12/E47 (E) antiserum and a 50-fold molar excess of SREm1 (m1) or SREm2 (m2). Arrows indicate the position of the specific complex, 'supershifted' or not, and of the free probe. **c**, Increase in specific binding during the course of differentiation. Extracts from myoblasts, growing (G) or after one day (D1) or two days (D2) of differentiation, were analysed by EMSA using the SREm2 oligonucleotide as a probe (E-box complex, lanes 1-6) or an SP1 binding site (5'-AGCTTGGGCGGGGCT-3', SP1 complex, lane 1'-5'), in the presence of a 50-fold molar excess of unlabelled SREm1 (m1) or SREm2 (m2) as indicated.

METHODS. Differentiation was induced as described in the legend to Fig. 1. Nuclear extracts from L6 $\alpha 1$ (**a** and **c**) or from the mouse muscle cell line Sol8²⁷ (which was used instead of L6 in **b** in order to optimize the recognition by the anti-mouse MyoD antiserum) were prepared according to ref. 28 and 10 μ g of extract was used in each lane. In **b**, 0.5 μ l antisera was added (incubation, 30 min, room temperature). EMSA analysis was according to ref. 9.

was always due to the more concentrated protein (Fig. 2d); no ternary complex could be detected by electrophoretic mobility shift assay in any condition tested (not shown). Mutation in the E-box was detrimental for E12/MyoD (Fig. 2e, lane 5) and E47/MyoD (not shown) binding, whereas mutation in the CARg box did not have any effect (lane 4).

Using SREm2 oligonucleotide (which does not bind SRF and thus simplifies the interpretation of data complicated by overlapping bands), E-box binding proteins were detected in nuclear extracts from muscle cells (Fig. 3a, lane 2). The E-box complex was not competed away by oligonucleotides corresponding to SREm1 (lane 3), to an AP-1-like element known to overlap with the SRE²³ (lane 6) or to an irrelevant oligonucleotide (lane 8). In contrast, the wild-type SRE (lane 4), the SREm2 (lane 5) or a shorter version of the SRE (lane 7) were good competitors. The E-Box complex contains proteins antigenically related to MyoD and to E12/E47 because part of the complex was supershifted with either an anti-MyoD or an anti-E12/E47 antiserum (Fig. 3b, lanes 4 and 5). The supershifted complex was competed away by an excess of unlabelled SREm2 oligonucleotide and not by an excess of SREm1 (lane 3 and 4), and did not appear when an anti-Myb antibody was used (lane 2) or in the absence of nuclear proteins (data not shown). A small but highly reproducible increase in the formation of the E-box-specific complex occurred during the course of myogenesis, when extracts

were analysed by comparison with SP1 DNA-binding activity (Fig. 3c).

We next compared the expression of various *c-fos* CAT promoter constructs transfected into myoblasts at day 1 of differentiation. Reporter expression was inhibited by the presence of the E-box, because the SREm1/CAT showed an increased level of expression when compared to the wild-type SRE reporter (Fig. 4a, compare lanes 3 and 2). This effect was not detectable in non-muscle cells treated the same way (transfected after 24 h in differentiation medium; Fig. 4a, lanes 6 and 7). This result suggests that, in muscle cells, negatively acting factors (for instance MyoD, see Fig. 3c) used the E-box as a target promoter sequence. To assess directly whether *c-fos* could be the target of MyoD-mediated downregulation, we did cotransfection experiments in cells lacking endogenous MyoD. SRE promoter activity was reduced on expression of MyoD (data not shown). In agreement with DNA-binding data, coexpression of MyoD together with E12 showed a more drastic effect (Fig. 4b, upper part). This inhibition was target-specific because overexpression of MyoD and E12 did not have any effect on an irrelevant promoter (Rous sarcoma virus long terminal repeat; data not shown). This inhibition requires MyoD/E-box interaction because it was not observed with a MyoD mutant lacking the DNA-binding domain (construct pEMSV-MyoD Δ ; Fig. 4b, lower part) which shows a complete response (100% of control

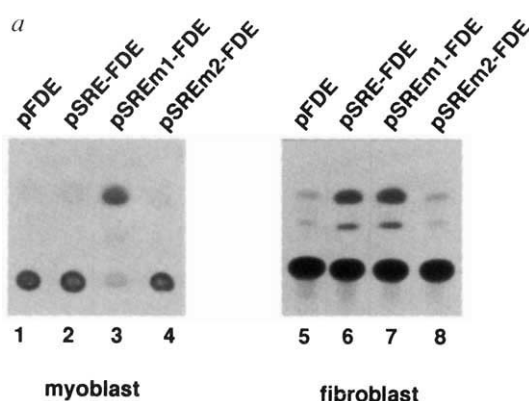
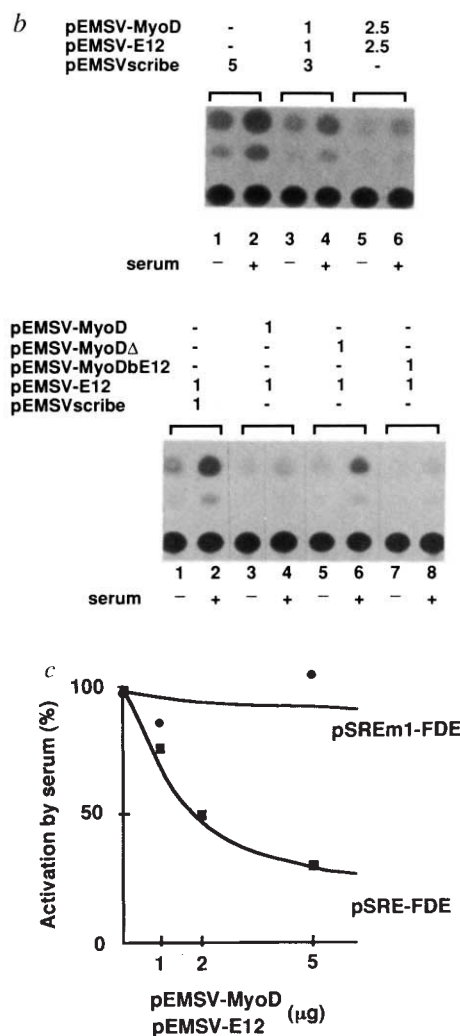


FIG. 4 Biological function of the *c-fos* E-box. *a*, The E-Box acts as a negative element in differentiating myoblasts. Myoblasts at day 1 of differentiation or fibroblasts (treated the same way) were transfected with the indicated CAT-reporter constructs. *b*, MyoD inhibits the SRE response to serum through its DNA-binding domain. Fibroblasts were transfected with pSRE-FDE and expression vectors (amounts in μg indicated above the lanes): pEMSV-E12 and pEMSV-MyoD²⁹ (wild-type MyoD, pEMSV Δ (D102-135²⁹) or pEMSB bE12 (E12²⁹), serum-deprived and treated as indicated. *c*, Inhibition requires an intact E-box on the *c-fos* promoter: pSRE-FDE (solid squares) or pSREm1-FDE (solid circles) response to serum when cotransfected with increasing doses of pEMSV-MyoD and pEMSV-E12. (100%, response when cotransfected with the non-expressor pEMSVscribe α 2).

METHODS. Plasmid pFDE includes the *c-fos* sequences from -99 to +42 subcloned into PGEM-CAT C. Plasmids pSRE-FDE, pSREm1-FDE and pSREm2-FDE have been constructed by inserting oligonucleotides into the *Xba*I site of pFDE. pEMSV-E12 was constructed by inserting a *Hind*III-*Eco*RI insert from pE12 into pEMSVscribe α 2. 5×10^6 myoblasts (L6 α 1) or fibroblasts (NIH3T3) were electroporated as described³⁰ with 2 or 5 μg of reporter construct. All cotransfection experiments included indicated doses of expression vector, completed to 5 μg with pEMSVscribe α 2 (non-expressing control vector). For serum response, cells were treated as described¹⁸. CAT assays were done as in ref. 31. For quantification, spots were excised and counted. Transfection efficiency was controlled using a pRSV-luc cotransfected vector (1 μg ; luciferase activity was measured using standard procedures on a Lumac luminometer) or by direct estimation of the intracellular plasmid³⁰.

response, mean of six experiments). Furthermore, an E-box-defective (pSREm1-FDE) reporter construct retained a normal response when the wild-type MyoD expression vector was used (Fig. 4c). A MyoD mutant in which the MyoD basic domain has been replaced by the E12 basic domain (pEMSV-MyoDbE12; Fig. 4b, lower part) retains a full inhibitory activity (30% of control response, mean of six experiments). This last result suggests a good correlation between *c-fos* promoter inhibition and cell proliferation inhibition.

Inhibition of *c-fos* by MyoD appears to involve a direct antagonism with SRF complexes for binding to the SRE (at least at the onset of differentiation, an alternative mechanism might take over this direct effect in the following steps). MyoD inhibitory effect on *c-fos* stands in contrast to its activating effect



on muscle gene promoters. This discrepancy might be due to the difference in context sequence between both systems (distinct basal promoter and surrounding enhancer sequences).

Note that muscle genes are usually under the control of a combination of CarG (SRF or rSRF binding sites) and E-boxes (basic helix-loop-helix binding sites), usually in close proximity²⁴. It is thus possible that MyoD is able to interact physically with SRF or rSRF²⁴, which would provide an alternate mechanism for SRF inhibition. In any case, MyoD seems to inactivate AP-1 at two levels: *c-Jun* protein absorption¹⁵ and *c-fos* gene transcription inhibition (this study). AP-1 inactivation could be, at least in part, responsible for cell proliferation inhibition by MyoD. □

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The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1

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MANY tyrosine kinases, including the receptors for hormones such as epidermal growth factor (EGF), nerve growth factor and insulin, transmit intracellular signals through Ras proteins¹⁻⁴. Ligand binding to such receptors stimulates Ras guanine-nucleotide-exchange activity⁵⁻⁹ and increases the level of GTP-bound Ras¹⁰⁻¹², suggesting that these tyrosine kinases may activate a guanine-nucleotide releasing protein (GNRP). In *Caenorhabditis elegans* and *Drosophila*, genetic studies have shown that Ras activation by tyrosine kinases requires the protein Sem-5/drak, which contains a single Src-homology (SH) 2 domain and two flanking SH3 domains¹³⁻¹⁵. Sem-5 is homologous to the mammalian protein Grb2, which binds the autophosphorylated EGF receptor and other phosphotyrosine-containing proteins such as Shc through its SH2 domain^{16,17}. Here we show that in rodent fibroblasts, the SH3 domains of Grb2 are bound to the proline-rich

carboxy-terminal tail of mSos1, a protein homologous to *Drosophila* Sos. Sos is required for Ras signalling¹⁸⁻²⁰ and contains a central domain related to known Ras-GNRPs²¹⁻²³. EGF stimulation induces binding of the Grb2-mSos1 complex to the autophosphorylated EGF receptor, and mSos1 phosphorylation. Grb2 therefore appears to link tyrosine kinases to a Ras-GNRP in mammalian cells.

To investigate whether mSos1 was a downstream target of Grb2, antibodies were raised to synthetic peptides derived from two different regions of mSos1. Both of these antibodies specifically bound a polypeptide with an apparent M_r of about 170K from mouse NIH3T3 fibroblasts overexpressing the human EGF receptor (EGFR) (see Fig. 1a). Although mSos1 has a predicted M_r of 150K, the immunizing peptide competed for binding (data not shown), confirming the identity of the immunoprecipitated protein; its abnormally low electrophoretic mobility may be due to its high proline content. Lysates from unstimulated or EGF-stimulated cells were immunoprecipitated with anti-Grb2 or anti-mSos1 antibodies, and these immune complexes were then analysed by western blotting with antibodies to mSos1 (Fig. 1a). mSos1 could be readily detected in anti-Grb2 immunoprecipitates from both stimulated and unstimulated cells, indicating that Grb2 and mSos1 form a constitutive complex. The mobility of mSos1 decreased slightly after growth factor stimulation (Fig. 1a), indicating that mSos1 may be phosphorylated on EGF stimulation. Treatment of anti-mSos1 immunoprecipitates from EGF-stimulated cells with potato acid phosphatase restored mSos1 mobility, indicating that EGF induces its phosphorylation (Fig. 1b).

Grb2 binds the autophosphorylated EGFR^{16,17}. To test whether mSos1 was recruited to the activated receptor through its association with Grb2, anti-EGFR immunoprecipitates from unstimulated and EGF-stimulated cells were blotted with anti-mSos1 antibodies. mSos1 associated with the EGFR was detected within 1 min of EGF stimulation (Fig. 1c), indicating that

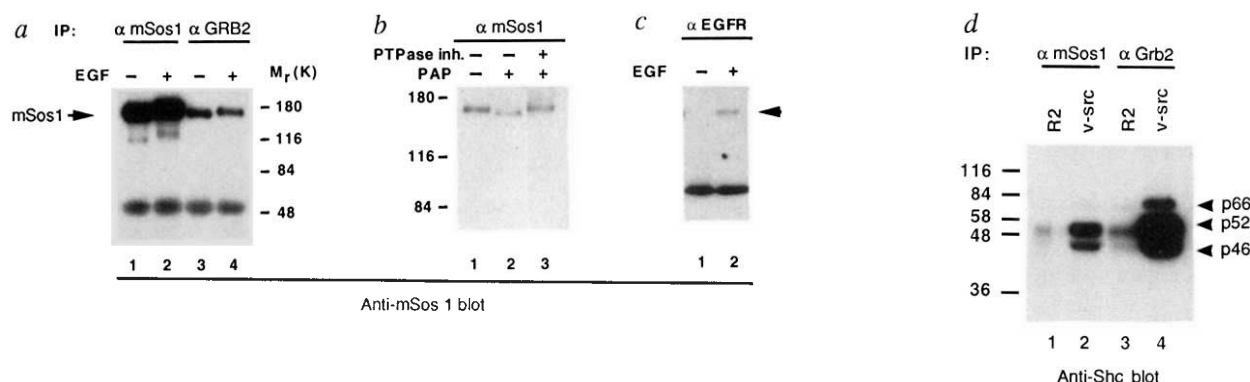
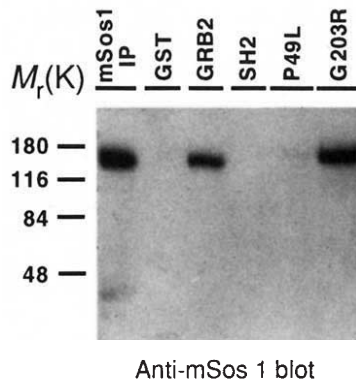


FIG. 1 Interactions of a Grb2-mSos1 complex with the EGFR and Shc proteins. *a*, mSos1 associates with Grb2. NIH3T3 cells overexpressing the human EGFR (HER14) were unstimulated (lanes 1 and 3) or were treated for 5 min with EGF (lanes 2 and 4). Cell lysates were immunoprecipitated with anti-mSos1 or anti-Grb2 antibodies as indicated, and were western blotted with anti-mSos1 antibodies. The position of mSos1 is indicated by an arrow. The mobilities of size markers are shown. *b*, mSos1 is phosphorylated. Anti-mSos1 immunoprecipitates from EGF-stimulated HER14 cells were untreated (lane 1) or were incubated with potato acid phosphatase (PAP) (lane 2) or with PAP and phosphatase inhibitor (lane 3). Samples were blotted with anti-mSos1 antibodies. *c*, mSos1 associates with activated EGFR. Anti-EGFR immunoprecipitates from serum-starved HER14 cells that were either unstimulated (–) or stimulated with EGF for 1 min (+) were blotted with anti-mSos1 antibodies. mSos1 is arrowed. *d*, mSos1 associates with Shc proteins in v-src-transformed cells. Rat-2 cells (R2) or v-src-transformed Rat-2 cells (v-src) were lysed, and immunoprecipitated with anti-mSos1 or anti-Grb2 antibodies. The immune complexes were then immunoblotted with anti-Shc antibodies.

METHODS. Serum-starved HER14 cells were stimulated for 5 min (*a*, *b*) or 1 min (*c*) with 80 nM EGF. *a*, Cells were lysed in PLC-lysis buffer and

immunoprecipitated with anti-Grb2 antibodies¹⁶ as previously described¹⁷. mSos1 was precipitated from 1 ml of lysate (protein concentration of 1 mg ml⁻¹) with 5 µl anti-mSos1 rabbit antiserum 1A, raised to a synthetic peptide spanning residues 1,241 to 1,260 in the C-terminal tail of mSos1 (ref. 18). Immune complexes were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-mSos1 antibodies followed by ¹²⁵I-protein A. *b*, Anti-mSos1 immunoprecipitates from EGF-stimulated HER14 cells were washed three times with HNTG buffer (20 mM HEPES pH 7.0, 150 mM NaCl, 0.1% Triton-X-100, 10% glycerol) and twice with phosphatase buffer (20 mM HEPES pH 7.0, 5% glycerol, 0.05% Triton-X100, 2.5 mM MgCl₂, 50 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin) and incubated with 1U of PAP in the absence or presence of 10 mM Na₃VO₄ as previously described³¹. mSos1 was identified by blotting as in *a*. *c*, Cell lysates were immunoprecipitated with anti-EGFR antibodies as previously described¹⁷, and blotted as in *a*. *d*, Confluent Rat-2 cells or v-src-transformed Rat-2 cells were lysed and immunoprecipitated with anti-mSos1 or anti-Grb2 antibodies as described above. Immune complexes were separated on an 8.25% SDS-polyacrylamide gel and blotted with affinity-purified anti-Shc antibodies as previously described¹⁷.

FIG. 2 Binding of Grb2 to mSos1 *in vitro* requires SH3 domains. Bacterially expressed glutathione S-transferase (GST) fusion proteins containing wild-type Grb2 (GRB2), mutant Grb2 proteins with amino-acid substitutions in the N-terminal SH3 domain (P49L) or C-terminal SH3 domain (G203R), or the Grb2 SH2 domain alone (SH2) were immobilized and incubated with lysates of HER14 cells. Associated proteins were immunoblotted with anti-mSos1 antibodies. Parental GST was included as a negative control. An anti-mSos1 immunoprecipitate (IP) is also shown. mSos1 is arrowed.



METHODS. Cell lysate (1 ml of 1 mg ml^{-1}) was incubated for 90 min at 4°C with about $5 \mu\text{g}$ of GST fusion proteins immobilized on glutathione-agarose beads, as previously described^{17,24}. Beads were washed three times in HNTG buffer, separated on a 7.5% SDS-polyacrylamide gel and blotted with anti-mSos1 antibodies. Wild-type and mutant GST-Grb2 fusion proteins have been described¹⁶.

mSos1 rapidly forms a complex with the autophosphorylated EGFR.

The v-Src tyrosine kinase signals through Ras proteins, but does not bind detectably to Grb2 (ref. 17). Phosphorylation of the Shc proteins in v-src-transformed cells induces their association with Grb2, however, suggesting a way in which v-Src might couple to the Ras pathway^{17,24}. Consistent with this possibility, when anti-mSos1 immunoprecipitates from v-src-transformed cells were analysed by western blotting with anti-Shc antibodies, prominent 46 and 52K bands were observed (Fig. 1d); mSos1-associated proteins with these mobilities contained phosphotyrosine (results not shown). These results indicate that tyrosine phosphorylation of Shc proteins by v-Src induces their

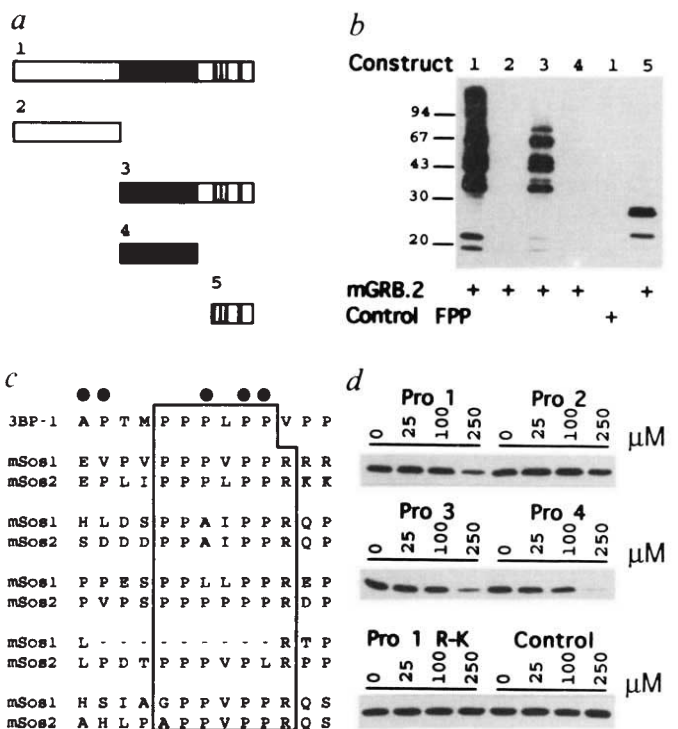
association with the Grb2-mSos1 complex.

Grb2 consists largely of a single SH2 domain and two SH3 domains^{16,32}. As the SH2 domain mediates binding to autophosphorylated growth factor receptors and tyrosine phosphorylated Shc proteins, we tested whether the SH3 domains of Grb2 interacted with mSos1, using bacterial GST-Grb2 fusion proteins. Whereas wild-type GST-Grb2 bound mSos1 in lysates of unstimulated cells, a mutant GST-Grb2 fusion protein with leucine substituted for Pro 49 in the amino-terminal SH3 domain (a mutation leading to loss of function in *C. elegans* Sem-5; ref. 13) failed to bind mSos1 (Fig. 2), suggesting that this domain is critical for association with mSos1. In contrast, substitution of Gly 203 with Arg in the C-terminal Grb2 SH3 domain had little effect on Grb2-binding, consistent with the much weaker phenotypic effect in *C. elegans* of the corresponding mutation in Sem-5 (ref. 13). The Grb2 SH2 domain alone had no mSos1 binding activity, consistent with the notion that it is the SH3 domains that recognize mSos1.

To delineate the binding sites for the Grb2 SH3 domains in mSos1, fragments of mSos1 were transcribed and translated *in vitro*, and tested for their ability to bind a GST-Grb2 fusion protein. Only fragments of mSos1 including the C-terminal tail of mSos1 bound (Fig. 3a, b). This region contains several conserved proline-rich motifs (Fig. 3c) resembling the core of a peptide (PPPVP; single-letter amino-acid code) that mediates binding of the protein 3BP1 to the SH3 domains of c-Abl and c-Src²⁵⁻²⁷. Synthetic peptides corresponding to each of the four proline-rich sequences in mSos1 (Pro 1-4) were used in competition experiments to test whether these sequences form part of a binding site for the Grb2 SH3 domains. Three of the four peptides were indeed able to compete for the binding of Grb2 to a fragment of mSos1 (Fig. 3d). Previous analysis of 3BP1 demonstrated that specific amino acids within, or N-terminal to, the proline-rich core are critical for binding to c-Abl and c-Src²⁷. The presence of an arginine C-terminal to each of the proline-rich sequences within the Sos proteins indicated that

FIG. 3 Mapping of the region of mSos1 which binds Grb2. a, Vectors encoding either the full-length mSos1 (1), or different subfragments which together encompass the full-length protein (2-5) were constructed. Vertical lines in the C-terminal region of mSos1 correspond to the proline-rich peptides in c, and the stippled box to the putative GNRP domain¹⁸. b, Binding of mSos1 to Grb2 requires the C-terminal tail of mSos1. The constructs indicated in a were transcribed and translated *in vitro* in the presence of ^{35}S -methionine, and incubated with GST-Grb2 fusion protein. Complexes were recovered using glutathione-agarose beads. Only radiolabelled mSos1 proteins which include the C-terminal tail bound specifically to the GST-Grb2 fusion protein. Multiple bands were obtained with the larger constructs because of internal initiation or cleavage of the translation products. Control FPP represents an irrelevant GST fusion protein. c, Sequences of proline-rich peptides in the C-terminal region of mSos1 and mSos2 proteins which resemble the core of a peptide required for binding of 3BP1 to c-Src and c-Abl SH3 domains^{25,27}. Solid circles indicate residues in 3BP1 which have been shown to be critical for its binding to c-Abl²⁷. Note also the highly conserved arginine residue in each of the mSos proline-rich peptides. d, Competition between an mSos1 C-terminal fragment and individual synthetic 11 amino-acid peptides for binding to Grb2. The peptides used correspond to the first 11 amino acids of each of the mSos1 proline-rich peptides shown in c (Pro 1, EVPPPPPPPP; Pro 2, HLDSPPAIPPP; Pro 3, PPSPPLPPPP; Pro 4, HSIAGPPVP). The radiolabelled mSos1 fragment used included only the most C-terminal proline-rich peptide. Only Pro 2 failed to compete significantly with the mSos1 fragment for binding to Grb2. Substitution of the conserved arginine in Pro 1 (Pro 1 R-K) abolished its ability to compete for Grb2 binding. A control peptide, corresponding to the N-terminal region of mSos1, failed to compete for Grb2-mSos1 binding.

METHODS. Constructs 1-5 encode amino acids 1-1,319, 1-598, 601-1,319, 601-1,019, and 1,132-1,319 of mSos1, respectively, where amino acid 1 corresponds to the methionine at position 18 in ref. 18. Constructs 1 and 2 used the mSos1 initiation methionine, and 3-5 an optimal consensus initiation sequence. These were *in vitro* transcribed and translated using a rabbit reticulocyte lysate system (Promega) in the presence of ^{35}S -methionine (Du Pont). *In vitro* translation products were incubated with about $2 \mu\text{g}$ of a GST-mouse Grb2 fusion protein in PLC lysis buffer on ice for 1 h,



complexes were recovered using glutathione-agarose beads (Sigma), washed three times in PLC-lysis buffer and then analysed by SDS-PAGE and autoradiography. Synthetic peptides used for competition studies were purified using HPLC. The fragment of mSos1 used for competition experiments corresponds to amino acids 1,204-1,319.

this residue might be important for binding to the Grb2 SH3 domains. Substituting a lysine for the arginine at this position indeed abolished the ability of the Pro 1 peptide to compete with mSos1 for binding to Grb2 (Fig. 3d). The arginine in this position may confer some specificity to the interaction between mSos1 and Grb2.

These results indicate that mSos1 is a physiological ligand for the Grb2 SH3 domains, consistent with the observations that the human counterpart of mSos1 ectopically expressed in 293 cells also complexes with Grb2 (ref. 28) and that Ras guanine nucleotide exchange activity is associated with Grb2 (ref. 29). Several mechanisms for the regulation of mSos1 activity can be envisaged. Binding of the Grb2-mSos1 complex to the autophosphorylated EGFR results in relocalization of mSos1 to the

particulate fraction of the cell (M.R.-A., unpublished results). Association of mSos1 with the membrane may bring this GNRP into contact with Ras, which is a membrane-associated protein, and thereby stimulate exchange of GDP for GTP. Binding of the Grb2 SH2 domain to a phosphotyrosine-containing site on the EGFR or Shc may also induce a conformational change which could be transmitted through the SH3 domains, modulating mSos1 GNRP activity or Grb2-binding. mSos1 apparently becomes phosphorylated after EGF stimulation. The identity of the phosphorylated residues, and the effect on mSos1 activity remain to be determined. In yeast, however, phosphorylation of the Ras exchanger Cdc25, apparently by cAMP-dependent protein kinase, triggers its relocalization from the membrane to the cytosol, terminating its interaction with Ras proteins³⁰. □

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Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling

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MANY of the actions of receptor tyrosine kinases are mediated by the protein Ras¹⁻⁵, including the activation of various downstream serine/threonine kinases and the stimulation of growth and differentiation⁶⁻¹². The human protein Grb2 binds to ligand-activated growth factor receptors and downstream effector proteins through its Src-homology (SH) domains SH2 and SH3, respectively^{13,14}, and like its homologue from *Caenorhabditis elegans*, Sem-5, apparently forms part of a highly conserved pathway by which these receptors can control Ras activity¹⁵⁻¹⁸. Here we show that the SH3 domains of Grb2 bind to the carboxy-terminal part of hSos1, the human homologue of the *Drosophila* guanine-nucleotide-releasing factor for Ras, which is essential for control of Ras activity by epidermal growth factor receptor and sevenless^{19,20}. Moreover, a synthetic 10-amino-acid peptide containing the sequence PPVPPR specifically blocks the interaction. These results indicate that the Grb2/hSos1 complex couples activated EGF receptor to Ras signalling.

To determine whether Grb2 associates with hSos1 *in vivo*, human kidney 293 cells were cotransfected with mammalian expression vectors which direct the synthesis of hSos1, Grb2 and the epidermal growth factor (EGF) receptor (EGFR). After 24 hours the cells were incubated with or without EGF and lysed. Proteins precipitated with anti-Grb2 or anti-EGFR antibodies were analysed by western blotting using antibodies against Grb2 or hSos1. Anti-hSos1 antibodies recognize a protein that migrates in SDS gels with an apparent M_r of 170K rather than the predicted M_r of 150K (ref. 18, and P.C. *et al.*, manuscript submitted). Cell lysates precipitated with anti-Grb2 antibodies and blotted with anti-hSos1 antibodies indicate that hSos1 is associated with Grb2 (Fig. 1a). Moreover, when the cotransfected EGFR was stimulated with EGF, the Grb2-hSos1 complex was found to associate with the activated EGFR (Fig. 1b). Similar results were obtained with the endogenous Grb2 and mSos1 proteins in NIH 3T3 cells (data not shown, and ref. 21). The SH2 domain of Grb2 is responsible for binding to the activated EGFR and other cellular proteins containing phosphotyrosine^{13,14,22}, and Y1068 of the EGFR functions as a high-affinity binding site for Grb2 when phosphorylated (A.B. *et al.*, manuscript in preparation). In response to EGFR autophosphorylation, the Grb2-hSos1 complex may therefore bind to the receptor through the SH2 domain of Grb2.

To determine the region(s) in hSos1 responsible for binding to Grb2, deletion mutants of hSos1 were generated as glutathione-S-transferase (GST)-fusion proteins in *Escherichia coli* and bound to glutathione beads. The beads were incubated with cell lysates containing Grb2, and the bound proteins were analysed by western blotting with anti-Grb2 antibodies. The results indicated that the C-terminal region of hSos1 was responsible for binding to Grb2. To identify the minimal region(s) in hSos1 responsible for binding, additional fusion proteins were generated, yielding the minimal binding site in construct VII (Fig. 2). In an alternative approach, random fragments of the hSos1

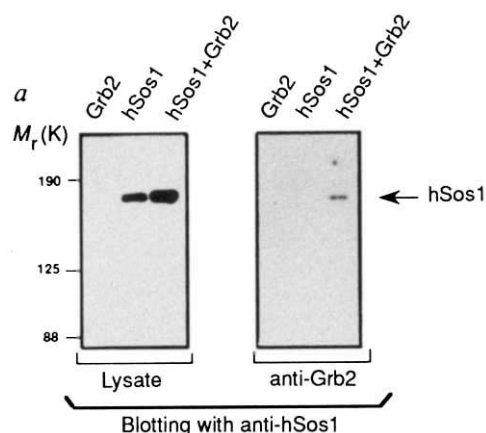
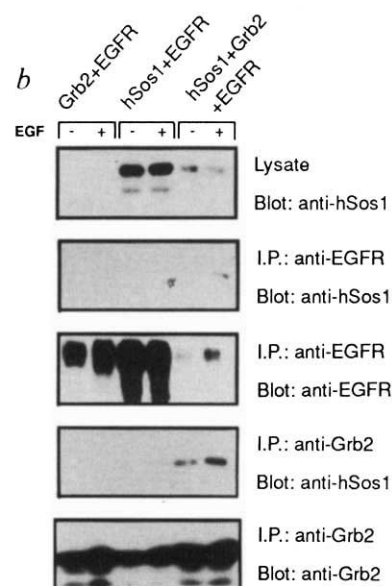


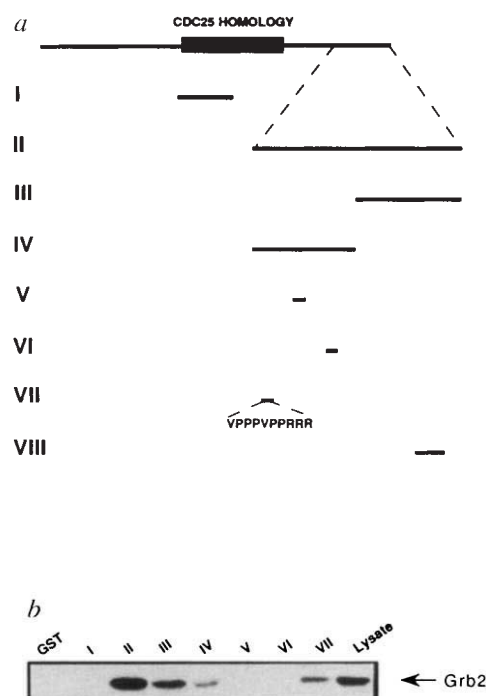
FIG. 1 Association between Grb2 and hSos1 in intact cells. Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors which direct the synthesis of hSos1, Grb2 and EGFR. **a**, The cells were transfected with hSos1, Grb2 or both together. Lysates from unstimulated cells were directly run on SDS gel or subjected to immunoprecipitation with anti-Grb2 antibodies. After SDS-PAGE, the samples were transferred to nitrocellulose and immunoblotted with anti-hSos1 antibodies. **b**, Cells were transfected with mammalian expression vectors encoding hSos1 and EGFR, Grb2 and EGFR or all three expression vectors together. Cells were then either mock treated or stimulated with EGF (250 ng ml⁻¹) for 2 min at 37 °C, lysed and immunoprecipitated and immunoblotted with different antibodies as indicated.

METHODS. Cells were transfected with a CMV-based eukaryotic expression vector using the calcium phosphate precipitation method, as previously described²⁹. Twenty-four hours after transfection, the cells were incubated in medium containing 0.5% fetal calf serum for an additional 16 h. The cells were treated with or without EGF, lysed, subjected to immunoprecipitation, run on an SDS-gel and immunoblotted according to published procedures³⁰.



Grb2 antibodies 50 and 86 were used for immunoprecipitation and immunoblotting, respectively¹³. The monoclonal antibody 108 was used for immunoprecipitation and anti-C anti-EGF-receptor antibodies were used for immunoblotting of EGFR-receptor³⁰. The anti-hSos1 antibodies are rabbit polyclonal antibodies directed against the catalytic domain of hSos1 (P.C. et al., manuscript submitted). Similar results were obtained with NIH 3T3 (HER14) cells which were stimulated with EGF and subjected to immunoprecipitation and western blotting with anti-Grb2 and anti-Sos1 antibodies (data not shown).

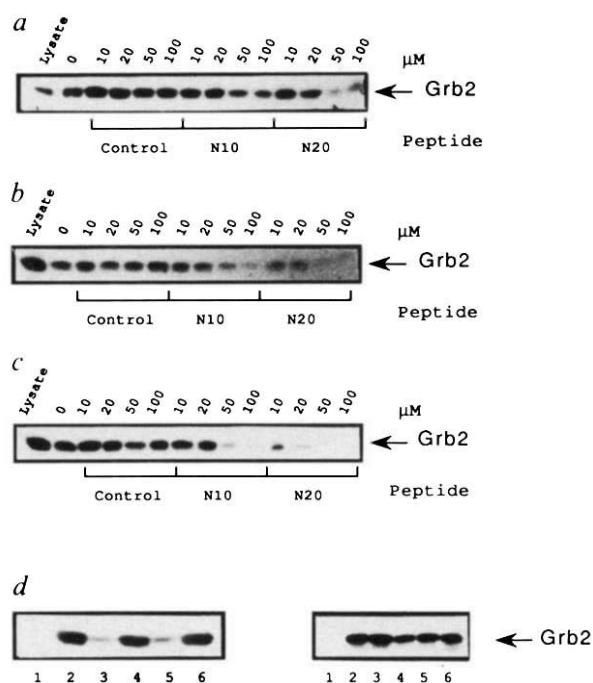
FIG. 2 Mapping the Grb2 binding sites on hSos1. **a**, Schematic representation of hSos1 and various deletion mutants used to delineate the Grb2 binding site on hSos1. Constructs I–VII represent glutathione-S-transferase (GST) fusion proteins. The amino acids shown represent the sequence of the peptide N10 that blocks binding of Grb2 to hSos1 (see Fig. 3) and which is contained within construct VII. Construct VIII represents a group of closely related T7 gene 10 fusion proteins that delineates a second binding site for Grb2 in the C-terminal region of hSos1. **b**, The binding of GST fusion hSos1 fusion proteins to Grb2. NIH 3T3 cell lysates (700 µg) were incubated with glutathione agarose beads carrying constructs I–VII or GST protein alone. After 2-h incubation, the beads were washed, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Grb2 antibodies. For comparison, 70 µg of NIH 3T3 lysate was loaded directly on the SDS-gel. **METHODS.** For constructs I, II, III and IV, oligonucleotide primers containing hSos1 sequences and appropriate restriction enzyme sites were synthesized and used to amplify DNA fragments from hSos1 complementary DNA template by polymerase chain reaction (PCR). The shorter constructs V, VI and VII were generated using overlapping oligonucleotides filled in with Klenow fragment. The DNA fragments generated were then digested with restriction enzymes and cloned into pGEX2T (Pharmacia). The resulting GST fusion proteins contain hSos1 residues 581–780 (I), 1,132–1,333 (II), 1,232–1,333 (III), 1,132–1,234 (IV), 1,176–1,187 LDSPPAIPPRQP (V), 1,207–1,219 PPSPPLLPREP (VI) and 1,147–1,159 VPVPPVPPRRRP (VII). All fusion proteins were purified by affinity chromatography on glutathione-agarose beads. About 2 µg of hSos1 fusion proteins (I–VII) or GST protein alone on beads were incubated with NIH3T3 cell lysates at 4 °C for 2 h and the beads were washed three times with HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). The slight differences in the total amount of Grb2 bound to constructs II, III, and IV is due to the variable proteolytic degradation of these deletion mutants. The T7 gene 10 fusion proteins of hSos1 were identified using the Novatope kit according to manufacturers instructions (Novagen). In brief, the C terminus of hSos1 was digested with DNaseI and cloned into the pTope vector. The bacteria containing the various constructs were then plated and the colonies lifted onto nitrocellulose. The bacteria were lysed and the nitrocellulose filters probed with biotinylated GRB2. Positive colonies were identified using an avidin-biotin sandwich



coupled to alkaline phosphatase (Vector Laboratory). The plasmids were then purified from the positive bacterial colonies and sequenced. Using this method several overlapping clones were identified which yielded a binding site encompassing the sequence from 1,289–1,313 of hSos1.

FIG. 3 Binding of Grb2 to hSos can be blocked by peptides from hSos1 and is mediated through the SH3 domains of Grb2. *a–c*, Synthetic peptides block the binding of Grb2 to hSos1. Cell lysates (700 μ g) were preincubated without or with different concentration of control peptide or peptides from the C-terminus of hSos1. The lysates were incubated with different hSos1 fusion proteins (Fig. 2) immobilized on glutathione-agarose beads. (*a*, construct II; *b*, construct III; *c*, construct IV.) The bound Grb2 protein was detected by immunoblotting with anti-Grb2 antibodies. The far left lane shows lysate (70 μ g) that was loaded directly onto the SDS-gel. *d*, Left, mutations in the SH3 domains, but not in the SH2 domain, reduce the binding of Grb2 to hSos1. The 293 cells were transfected with vector alone (lane 1), or various Grb2 constructs (wild-type, lane 2; SH3 mutant P49L, lane 3; SH2 mutant S90N, lane 4; SH3 mutant G203R, lane 5; SH2 mutant R86K, lane 6). The lysates were incubated with hSos1 GST fusion protein (construct II) immobilized on glutathione-agarose beads and the binding of the various constructs determined by immunoblotting with anti-Grb2 antibodies as in Fig. 2; Right, one sixth of the lysates used were loaded directly on the gel and subjected to immunoblotting with anti-Grb2 antibodies demonstrating that the expression of the different Grb2 constructs were similar.

METHODS. *a–c*, Three peptides of hSos1 were synthesized: control peptide QRKKIARDNGPGHNTFQSS (residues 738–757), the N20 peptide GTDEVPPPPVPPRRRPESA (residues 1,143–1,162) and the N10 peptide VPPVPPRRR (residues 1,149–1,158). The methods were identical to those described in legend to Fig. 2 except the lysates were preincubated with various concentrations of the peptides. *d*, Wild-type and mutant Grb2 proteins were subcloned into the expression vector and expressed in 293 cells as described in Fig. 1. After transfection for 24 h, cell lysates were incubated with 2 μ g GST hSos1 fusion protein (construct II) for 2 h at 4 °C. The beads were washed with HNTG and immunoblotted with anti-Grb2 antibodies. The loss-of-function amino-terminal SH3 mutant P49L (lane 3) and C-terminal mutant G203R (lane 5) were previously described^{13–15,23}.



gene were generated by DNase I digestion, expressed as T7 gene 10 fusion proteins and screened for binding to Grb2; the binding site in construct VIII (Fig. 2a) was identified. Both sequences are rich in proline residues and resemble a motif in the protein 3BP1 responsible for binding the Abl SH3 domain²³. We therefore tested whether short synthetic peptides derived from construct VII (Fig. 2) interfered with the association between Grb2 and the hSos1 fusion proteins. A 10-amino-acid peptide containing the sequence PPVPPR specifically blocked hSos1 binding to Grb2. The sequence motif PPVPPR (where Ψ denotes a hydrophobic amino acid) is conserved in *Drosophila* and mammalian Sos (refs 18–20, and P.C. *et al.*, manuscript submitted).

To identify the region in Grb2 responsible for binding to hSos1, lysates from human kidney 293 cells expressing various Grb2 mutant proteins were incubated with a GST hSos1 fusion protein capable of binding to Grb2 (construct II in Fig. 3) for two hours, washed, and analysed by western blotting with Grb2 antibodies. A Grb2 protein containing a point mutation in the SH2 domain that interferes with binding to tyrosine phosphorylated moieties^{13,14,22} bound to hSos1 as well as wild-type Grb2 (Fig. 3d), indicating that the SH2 domain is not required for binding. In contrast, mutations in either SH3 domain corresponding to loss-of-function *sem-5* alleles^{13–15} reduced binding, indicating that high-affinity binding requires the coordinate interaction of both Grb2 SH3 domains with the two PPVPPR motifs in the C terminus of hSos1. Real-time kinetic analysis of direct binding experiments using the BioSensor system (BIAcore Pharmacia)²⁴ shows that Grb2 binds to a synthetic peptide containing the PPVPPR motif with a dissociation constant (K_d) of 25 nM (M. Zhou *et al.*, unpublished results).

hSos1 possesses guanine nucleotide-releasing activity for Ras and its catalytic domain can rescue CDC25 in yeast (P.C. *et al.*, manuscript submitted). Moreover, genetic analysis indicates that the *Drosophila* homologue of Grb2 is essential for signalling by receptor tyrosine kinases and lies along the same signalling pathway as the *Drosophila* Sos and Ras proteins^{19,20,26,27}. We have shown that overexpression of Grb2 potentiates the capacity of EGF to activate Ras²⁵, and that this effect is mediated by an

increase in guanine-nucleotide-releasing activity²⁵. Overexpression of Grb2 also potentiates the ability of insulin to activate mitogen-activated protein (MAP) kinase and the insulin-induced increase in MAP kinase activity requires Ras activity (E.S. *et al.*, submitted). Taken together, these results indicate that the interaction between Grb2 and hSos1 is responsible for enhancing the rate of nucleotide exchange on Ras. This might result from activation of hSos1 on binding of the Grb2-hSos1 complex to the EGFR either by phosphorylation or by an induced conformational change. Alternatively, binding of the Grb2-hSos1 complex to the autophosphorylated EGF receptor may simply relocate the guanine nucleotide releasing activity from the cytoplasm to the membrane where Ras protein is located. Grb2 does not appear to be phosphorylated in response to EGF, insulin or cellular transformation by v-Src, indicating that it functions as an adaptor protein linking receptor tyrosine kinases to downstream effector proteins^{13,14,22}. The reduced electrophoretic mobility²¹ of Sos1 in stimulated cells is due to serine/threonine phosphorylation (N.L. *et al.*, unpublished). It has been recently shown that the phosphorylation of CDC25 in yeast leads to its dissociation from Ras²⁸.

The remarkable structural and functional conservation of Grb2 in *C. elegans*^{15–17}, *Drosophila*^{19,20,26,27} and mammals^{13,14,22} indicates that the interaction between hSos1 and the SH3 domains of Grb2 represents an important step in a major evolutionarily conserved signalling pathway for the control of cell growth and differentiation. □

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Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras

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ACTIVATION of receptor tyrosine kinases such as those for epidermal growth factor (EGF), platelet-derived growth factor, or nerve growth factor converts the inactive, GDP-bound form of Ras to the active, GTP-bound form^{1–4}, and a dominant negative mutant of Ras interferes with signalling from such receptors^{5–8}. The mechanisms by which receptor tyrosine kinases and Ras are coupled, however, are not well understood. Many cytoplasmic proteins regulated by such receptors contain Src-homology (SH) 2 and 3 domains, and the SH2- and SH3-containing protein Grb2, like its homologue from *Caenorhabditis elegans*, Sem-5, appears to play an important role in the control of Ras by receptor tyrosine kinases^{9–11}. Here we show that overexpression of Grb2 potentiates the EGF-induced activation of Ras and mitogen-activated protein kinase by enhancing the rate of guanine nucleotide exchange on Ras. Cellular Grb2 appears to form a complex with a guanine-nucleotide-exchange factor for Ras, which binds to the ligand-activated EGF receptor, allowing the tyrosine kinase to modulate Ras activity.

To study the effect of EGF on the activation of Ras we used NIH3T3 cells overexpressing the EGF receptor (HER14). The guanine nucleotides bound to Ras were measured by labelling cells with [³²P]phosphate, immunoprecipitating Ras with the monoclonal antibody Y13-259 (ref. 12) and analysing the bound nucleotides by thin-layer chromatography. EGF induces a rapid twofold increase in the proportion of GTP-bound Ras (Fig. 1a). This increase is higher than that observed in earlier studies^{1,13}, probably because of the overexpression of the EGF receptors in HER14 cells. The increase is transient: GTP-bound Ras levels are maximal within 2 min of EGF addition and returns to normal within 15 min. The protein Grb2 associates with tyrosine phosphorylated EGF receptor¹⁰ and its homologue from *C. elegans*, Sem-5, is crucial for growth factor control of Ras signalling⁹. To investigate whether the EGF-induced activation of Ras is mediated by Grb2, we compared the effects of EGF on the amount of GTP-bound Ras in HER14 cells and in HER14 cells expressing 20-fold more Grb2 (Grb2-HER14 cells) (Fig. 1b). These cell lines expressed similar amounts of Ras (Fig. 1c) and EGF receptors (Fig. 1d), and their basal levels of GTP-bound Ras were essentially identical. The EGF-induced increase in the proportion of GTP-bound Ras was approximately doubled in Grb2-HER14 cells, however, and persisted for at least 15 min.

Analyses of five additional Grb2-overexpressing cell lines showed a similar potentiating effect of Grb2 overexpression on the growth-factor-induced increase in the proportion of GTP-bound Ras.

To determine whether this effect on Ras activation is transduced to events downstream of Ras, we examined the effects of Grb2 overexpression on MAP kinase activation (not shown). Changes in mitogen-activated protein (MAP) kinase activity can be assessed by the appearance of an activated form with reduced electrophoretic mobility produced by threonine and tyrosine phosphorylation^{14,15}. On a western blot of lysates prepared from HER14 and Grb2-HER14 cells, an anti-MAP kinase antiserum recognized two polypeptides with *M_s* of 42 and 44K, corresponding to p42MAP2 kinase and p44MAP1 kinase, respectively (Fig. 2). Within 5 min of stimulation with EGF, the slower-moving form of both polypeptides represented 15% of the total in HER14 cells and 50% in Grb2-HER14 cells, indicating that Grb2 overexpression potentiates the EGF-induced activation of MAP kinases. This effect is probably mediated by Ras, as the activation of MAP kinase by EGF is dependent on Ras activation^{8,16}.

The activity of Ras is tightly regulated by GTPase-activating proteins (GAPs), which act by stimulating its intrinsically low GTPase activity (reviewed in ref. 17), and guanine-nucleotide-exchange factors, which act by promoting the exchange of bound GDP molecules for GTP (reviewed in ref. 18). To determine how Grb2 potentiates the EGF-induced activation of Ras, we examined the effects of EGF on GAP activity and on the exchange rate of Ras-bound nucleotides in HER14 cells and in Grb2-HER14 cells.

GAP activity was measured by incubating cell lysates with recombinant H-ras complexed with [α -³²P]GTP and quantifying the proportion of GTP-bound Ras. Lysates of HER14 cells and Grb2-HER14 cells had similar GAP activity, which was not affected by treating the cells with EGF for 5 min before lysis (Fig. 3a and b), indicating that induced activation of Ras in HER14 cells and its potentiation by Grb2 overexpression are not mediated by reduced GAP activity. To measure the rate of nucleotide exchange on Ras, cells were stimulated with EGF and then permeabilized with streptolysin-O in the presence of [α -³²P]GTP. At various intervals, cells were lysed, Ras was recovered by immunoprecipitation, and the binding of the labelled nucleotide to Ras was quantified by thin-layer chromatography or scintillation counting. In HER14 cells EGF treatment roughly doubled the rate of nucleotide binding to Ras, indicating that EGF activates Ras by stimulating guanine nucleotide exchange (Fig. 3c). In Grb2-HER14 cells, EGF treatment increased the rate of nucleotide binding to Ras about fourfold. The differences between HER14 and Grb2-HER14 cells did not reflect differences in the efficiency of permeabilization and/or the uptake of labelled nucleotide in the two cell lines, as the binding of [α -³²P]GTP to total cellular proteins in both cell types was identical, and was unaffected by EGF treatment (results not shown). EGF thus appears to stimulate the rate of guanine nucleotide exchange on Ras, and the effects of Grb2-overexpression indicate that this stimulation may be mediated by Grb2.

The SH2 domain of Grb2 mediates its interaction with acti-

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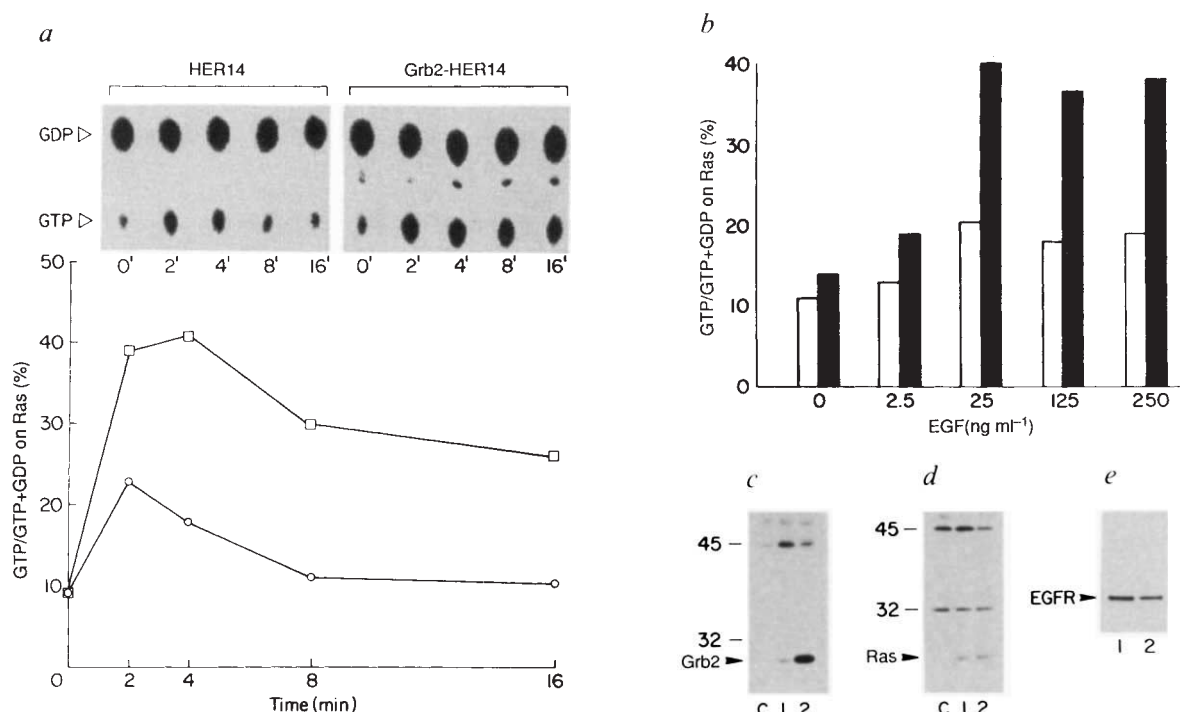


FIG. 1 Effect of Grb2 overexpression on EGF-induced Ras activation. **a**, Time courses of EGF-induced increase in GTP-bound to Ras in HER14 cells ($\circ$) and in Grb2-HER14 ($\square$) cells. The Ras was immunoprecipitated with the Ras-specific monoclonal antibody Y13-259 (ref. 12) from cells labelled with ^{32}P -orthophosphate that had been stimulated for the indicated times with 250 ng ml^{-1} EGF. Nucleotides were eluted, separated by thin-layer chromatography and quantified using a phosphorimager. The thin-layer chromatogram corresponding to the quantitative data is shown in the inset. The positions of GDP and GTP standards are indicated. **b**, EGF concentration dependence of the nucleotides bound to Ras in HER14 cells (open bars) and in Grb2-HER14 cells (black bars). ^{32}P -labelled cells were incubated with the indicated concentrations of EGF for 2 min. At the end of the incubation, Ras was immunoprecipitated and the amounts of nucleotides bound to Ras were quantified as in **a**. **c–e**, Expression of Grb2, Ras and EGF receptor in HER14 cells and in Grb2-HER14 cells. **c**, Grb2 was immunoprecipitated using anti-Grb2 serum¹⁰. **d**, Ras was immunoprecipitated using anti-Ras serum. **e**, Western blot of EGF receptor. Protein (50 μg) from each cell line was electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose sheet. The nitrocellulose sheet was incubated with anti-EGF receptor antisera²⁶, washed and incubated with ^{125}I -protein A. Immunoprecipitations were done as described²⁷ from lysates of cells labelled with ^{35}S -methionine. Immunoprecipitates were analysed on 12.5% polyacrylamide gels. Migration of molecular size standards is indicated at left in **k**. Lanes 1, HER14 cells; 2, HER14 cells overexpressing Grb2; C, control immunoprecipitation done in HER14 cells by using antibodies that were blocked by preincubation with an excess of purified antigen. Anti-Grb2 serum was incubated with 5 μg of purified Grb2 fusion protein (**c**) and anti-Ras serum was incubated with 5 μg of purified human H-ras protein (**d**).

METHODS. The Grb2 overexpressing cell line was established as follows. The EcoRI 1 kilobase (kb) fragment, which contains the entire Grb2 coding region, was cloned into pCMV expression vector. This clone and a plasmid that confers resistance to hygromycin (pGC47) were cotransfected into the NIH3T3 cell line overexpressing the EGF receptor HER14 (ref. 28) by the

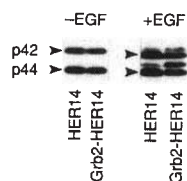
CaPO₄ precipitation method. The Grb2 overexpressing cell line was selected on the basis of hygromycin resistance and was methotrexate amplified in culture for ~6 months under increasing concentration of methotrexate ranging from 50 nM to 6.4 μM . Cells were cultured routinely in DMEM supplemented with 10% calf serum (CS) and 800 nM methotrexate. For serum starvation, cells were cultured in DMEM plus 0.5% CS for 48 h. Stimulation with EGF was done in DMEM containing 1 mg ml⁻¹ bovine serum albumin and 20 mM HEPES, pH 7.5. For the Ras activation assay, serum-starved cells were labelled with [^{32}P] orthophosphate (0.5 mCi ml⁻¹) for 12 h in phosphate-free DMEM supplemented with 0.5% phosphate-free CS. After treatment with EGF, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 5 min in lysis buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl₂, 1 mg ml⁻¹ BSA, 1% aprotinin, 100 $\mu\text{g ml}^{-1}$ leupeptin, 10 $\mu\text{g ml}^{-1}$ pepstatin, 100 mM benzamide, and 10 $\mu\text{g ml}^{-1}$ soybean trypsin inhibitor. Lysates were spun for 3 min at 14,000g to remove nuclei and supernatants were brought to 500 mM NaCl, 0.5% deoxycholate and 0.05% sodium dodecyl sulphate (SDS). Excess free nucleotides were removed by incubation with activated charcoal for 5 min. Samples were spun for 5 min at 14,000g to pellet charcoal. Immunoprecipitations were done for 40 min using 3 μg per sample of the rat monoclonal anti-Ras antibody Y13-259 precoupled to protein A-sepharose beads via rabbit anti-rat immunoglobulin antibody. Duplicate samples in which the Y13-259 antibody was omitted were used as controls. The immune complexes were collected by centrifugation and washed 8 times with a buffer containing 50 mM HEPES, pH 7.4, 500 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, and 0.005% SDS. Bound nucleotides were eluted with 2 mM EDTA, 2 mM DTT, and 0.2% SDS for 20 min at 68 °C. Nucleotides were separated by thin layer chromatography on PEI-cellulose plates in 0.75M KH₂PO₄, pH 3.5. Plates were imaged and quantified using a phosphorimager. A factor of 1/2 was applied to the counts obtained from GDP and a factor of 1/3 to those obtained from GTP as a correction for their respective phosphate content. Radioactivity in GTP or GDP from control samples did not exceed 10% of that measured in experimental samples.

vated receptors¹⁰. Much less is known about the function of the SH3 domains, although the SH3 domains of Abl and Src oncogenes bind to a proline-rich motif (PPPLPP; single-letter amino-acid code) in the protein 3BP-1^{19,20}. This sequence motif is also present in the C-terminal domain of mammalian and *Drosophila* Sos, a putative guanine-nucleotide-exchange factor for Ras (refs 21, 22, and P. Chardin, J. H. Camonis and N.W.G., manuscript in preparation), suggesting that the SH3 domains of Grb2 may mediate its interaction with a guanine nucleotide exchange factor for Ras. We therefore precipitated Grb2 from HER14 cells using anti-Grb2 antibody¹⁰, and assessed the ability of the immune complexes to stimulate [α - ^{32}P]GTP binding to

recombinant H-ras. The rate of binding was significantly stimulated by the complexes, indicating that they contained a guanine nucleotide exchange activity for Ras (Fig. 4a). The rate of dissociation of [α - ^{32}P]GDP bound to recombinant H-ras was also increased by the Grb2 immune complexes (Fig. 4a, inset). Under the same conditions, the complexes had no effect on the rate of [α - ^{32}P]GTP binding to the Ras-related protein RaL²³. As reported elsewhere in this issue, Grb2 binds to the mouse or human homologues of Sos^{24,25}. Moreover hSos1 is a guanine nucleotide exchange factor for Ras (P. Chardin, J. H. Camonis and N.W.G., manuscript in preparation). These observations suggest that the guanine-nucleotide-exchange activity detected

FIG. 2 Effect of Grb2 overexpression on EGF-induced activation of MAP kinase. Cells were treated with EGF (250 ng ml⁻¹) for 5 min and MAP kinase polypeptides were detected by western blotting of total cell lysates with anti-MAP kinase antibodies. The slower migrating forms of p42MAP2 kinase and p44MAP1 kinase correspond to activated forms of the kinases.

METHODS. HER14 and Grb2-HER14 cells were starved in 0.5% calf serum for 48 h and treated with EGF as indicated. Cells were lysed and 50 µg of total lysates were electrophoresed on a 12.5% polyacrylamide gel, blotted onto nitrocellulose and incubated with anti-MAP kinase antiserum 691 (ref. 15), immune complexes were detected by HRP-conjugated goat anti-rabbit antibodies followed by enhanced chemiluminescence detection (Amersham). The relative intensities of the MAP kinase bands were measured by densitometric scanning of the autoradiogram. Results shown are representative of data obtained in three experiments.



in Grb2 immune complexes is at least in part mediated by Sos proteins.

To test the effects of EGF stimulation on the Grb2-associated guanine nucleotide exchange activity, Grb2 was immunoprecipitated from lysates of serum-starved HER14 cells incubated with or without EGF. Similar levels of guanine-nucleotide-exchange activity were associated with Grb2 immune complexes in both resting and stimulated HER14 cells (Fig. 4b). As similar amounts of Grb2 were precipitated from resting and stimulated cells (results not shown), the absence of EGF-induced changes in the Grb2-associated guanine nucleotide activity could not have been due to alterations in the quantity of Grb2 present. Grb2 thus appears to interact constitutively with a guanine-nucleotide-exchange factor for Ras, a possibility supported by the report elsewhere in this issue that similar amounts of Grb2-mSos1 complex can be detected in cells before and after EGF stimulation²⁵.

As EGF stimulation induces Grb2 binding to the EGF receptor¹⁰, we investigated the possibility that guanine nucleotide-

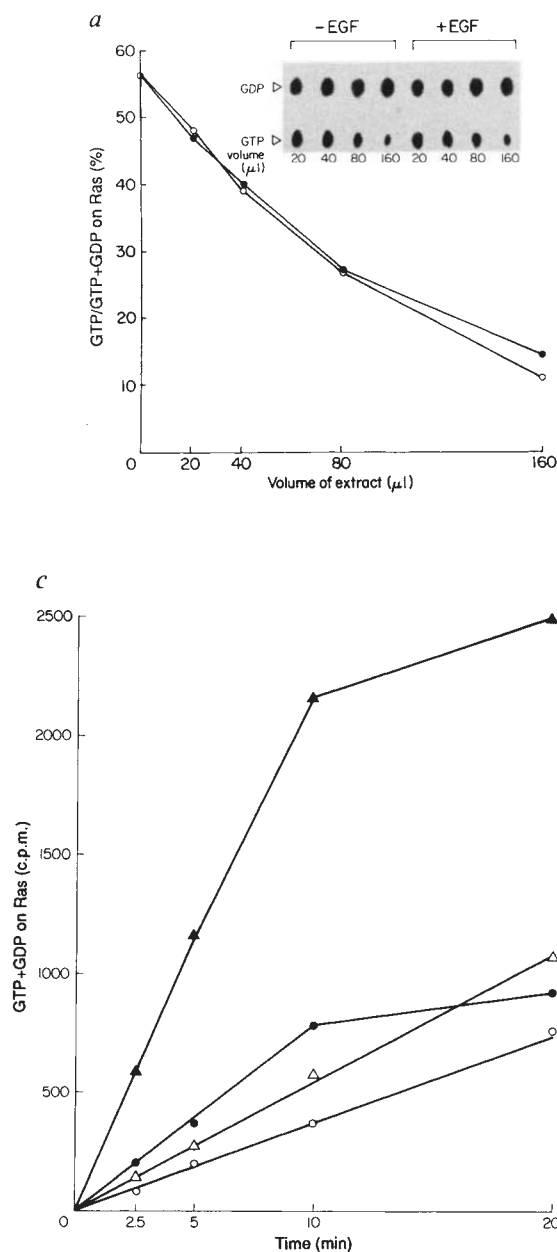


FIG. 3 Effect of EGF on GAP activity and on nucleotide binding to Ras in HER14 cells and in Grb2-HER14 cells. GAP activity was measured in detergent extracts from quiescent (○) or EGF-treated (●) HER14 cells (a) or Grb2-HER14 cells (b). Insets show the thin layer chromatogram corresponding to the quantitative data. c, The binding of [³²P]-labelled nucleotide to Ras was determined in permeabilized cells. Binding in quiescent (○) and EGF-stimulated (●) HER14 cells and in quiescent (△) and EGF-stimulated (▲) Grb2-HER14 is shown.

METHODS. Cells were maintained and serum starved as in Fig. 1. Five min before stimulation all cells were treated with 100 nM okadaic acid in stimulation medium. To assay for GAP activity, cells were stimulated with EGF for 2 min (250 ng ml⁻¹), rinsed twice with PBS, scraped in PBS residue, pelleted and then lysed in a buffer consisting of 20 mM Tris-Cl, pH 8, 0.5% NP-40, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 5 mM MgCl₂, 1 mM PMSF, 100 nM okadaic acid, 1% aprotinin, 100 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ pepstatin, 100 mM benzamidin, and 10 µg ml⁻¹ soybean trypsin inhibitor. Nuclei were removed by centrifugation at 15,000g for 5 min and supernatants (10 mg ml⁻¹) were assayed for GAP activity. Purified bacterially expressed human H-ras (500 ng) was incubated with 0.5 mCi [³²P]GTP (3,000 Ci mmol⁻¹) in 20 mM Tris-Cl pH 8, 5 mM EDTA and 2 mM DTT in a volume of 250 µl for 20 min on ice. After the addition of 10 mM MgCl₂, 2 mM DTT, 1 mM GTP and 1 mM GDP, 50 ng aliquots were added to varying amounts of the GAP extract made up to the same volume with lysis buffer. The reaction mixtures were incubated 30 min at room temperature and stopped

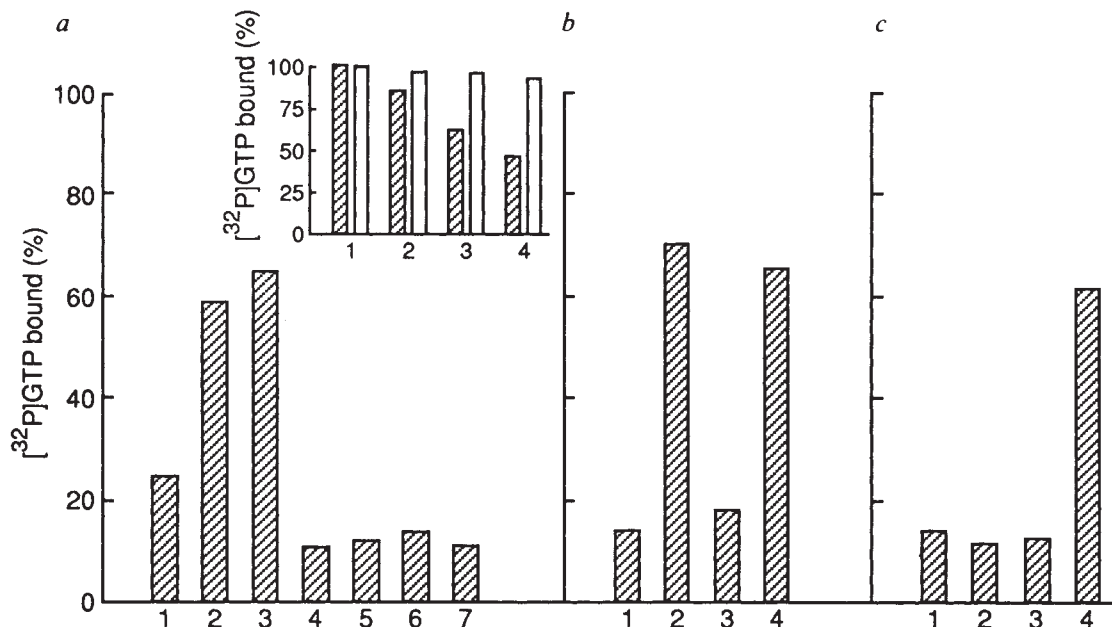


FIG. 4 Interactions of guanine nucleotide exchange factor for Ras with Grb2 and with the EGF receptor. Lysates from HER14 cells were immunoprecipitated with anti-Grb2 or with anti-EGF receptor antibodies. Immune complexes were washed and then incubated with recombinant H-ras and $[\alpha\text{-}^{32}\text{P}]\text{GTP}$. The amount of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ bound was determined by nitrocellulose filter binding assay. **a**, Binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to H-ras after incubations with anti-Grb2 immunoprecipitates for 10, 20 and 30 min (bars 1, 2 and 3, respectively); after 20 min incubations with immunoprecipitates obtained with non-immune (control) serum (bar 4), with protein A sepharose beads (bar 5) or with immunoprecipitates obtained with anti-Grb2 antibodies that were blocked by preincubation with an excess of purified Grb2 (bar 6). The binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to RalA after incubation with anti-Grb2 immunoprecipitates is shown in bar 7. Inset, Release of $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ from H-ras following incubations with anti-Grb2 immunoprecipitates (hatched bars) or control immunoprecipitates (open bars) for 10, 20 and 30 min (bars 1, 2 and 3, respectively). **b**, Binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to H-ras after incubation with anti-Grb2 immunoprecipitates (bars 2 and 4) or with control immunoprecipitates (bars 1 and 3) from serum-starved (bars 1 and 2) or from EGF-treated (bars 2 and 4) HER14 cells. **c**, Binding of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ to H-ras after incubation with anti-EGF receptor immunoprecipitates (bars 2 and 4) or with control immunoprecipitates (bars 1 and 3) from serum-starved (bars 1 and 2) or from EGF-treated (bars 2 and 4) HER14 cells.

METHODS. Cells were maintained and serum starved as in Fig. 1. For growth factor stimulation, cells were stimulated with 250 ng ml⁻¹ EGF for 5 min. For immunoprecipitations, HER14 cells were lysed in buffer containing 10 mM

Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1% aprotinin, 1 mM PMSF, 10 $\mu\text{g ml}^{-1}$ leupeptin, 1 mM sodium orthovanadate, 100 mM sodium fluoride and 10 mM sodium pyrophosphate. Lysates were clarified by centrifugation and immunoprecipitations were done for 90 min at 4 °C using anti-Grb2 antibodies or anti-EGF receptor antibodies and protein A-sepharose beads. Where indicated, anti-Grb2 antibodies were omitted, substituted with non-immune serum or blocked by preincubation with 5 μg of purified GST-Grb2. Immunoprecipitates were washed 10 times with binding buffer A (20 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 1 mM DTT, 10 mM NaCl and 50 $\mu\text{g ml}^{-1}$ BSA) and then resuspended in 25 μl of the same buffer containing 50 fmol purified H-ras or RalA proteins. Bacterially expressed H-ras and RalA were purified by column chromatography as described^{23,27}. The reaction was started at 32 °C by the addition of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (0.05 μCi , 3,000 Ci mmol⁻¹). At the indicated time, the reaction mixtures were centrifuged at 10,000g for 1 min to remove protein A-sepharose beads and the supernatants were filtered through nitrocellulose. Radioactivity associated with the filters was quantified by Phosphorimager. The ability of RalA to bind GTP was confirmed by incubating the protein with $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ in the presence of EDTA (5 mM). For the measurement of $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ release, purified H-ras was prebound to $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ (5 μCi per pmol) by incubation for 30 min at 30 °C in binding buffer A. 25- μl aliquots (50 fmol) of the prebound H-ras were added to the washed immunoprecipitates in the presence of non-radioactive GTP (500 μM). At the indicated intervals, immune complexes were pelleted and the amount of $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ remaining bound to H-ras was determined by nitrocellulose filter binding assay as described. Results are expressed as the percentage of maximal counts bound to the protein in the presence of EDTA (5 mM). The results shown are representative of at least 2 independent experiments.

by the addition of 4 vols cold lysis buffer containing 500 mM NaCl and 0.005% SDS. Ras was immunoprecipitated with Y13-259 antibody as in Fig. 1. Nucleotides bound to Ras were eluted and resolved by TLC as described in Fig. 1. To assay for exchange activity, subconfluent cultures of serum-starved HER14 cells and Grb2-HER14 (10-cm dishes) were stimulated with EGF (250 ng ml⁻¹). Cells were washed twice with PBS (37 °C) and to each dish 2 ml of permeabilization buffer consisting of 12.5 mM PIPES pH 7.4, 150 mM KCl, 37.5 mM NaCl, 6.25 mM MgCl₂, 1.25 mM ATP, 1 mM EGTA, 0.8 mM CaCl₂, 1 mM Na₂VO₄, 0.4 IU Streptolysin-O (Wellcome Diagnostic), and 50 $\mu\text{Ci ml}^{-1}$ $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (3,000 Ci mmol⁻¹, NEN) were added. At the indicated intervals, the permeabilization buffer was removed, cells were lysed and Ras was recovered by immunoprecipitation as described in Fig. 1, with the exception that the clarification step with activated charcoal was omitted. Labelled nucleotides bound to Ras were eluted as described in Fig. 1, and quantified by scintillation counting or by thin layer chromatography. Of the total nucleotides bound to Ras, 50% consisted of GTP and there was no significant change in this ratio throughout the course of the assay. For the measurement of total guanine nucleotide binding activity, 25 μl were removed from cell lysates before immunoprecipitation and spotted on nitrocellulose filter. The filters were washed 5 times with excess volume of ice-cold wash buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl and 3 mM MgCl₂). Results shown are the averages of two experiments and data was normalized to protein concentration.

exchange activity will also associate with the receptor under these conditions. EGF-receptor was immunoprecipitated from resting and EGF-stimulated cells, and the guanine nucleotide-exchange activity in the precipitates was measured. Precipitates from EGF-treated cells contained a significant amount of guanine nucleotide-exchange activity, whereas those from unstimulated cells contained none (Fig. 4c). These findings are consistent with a model in which activation of the EGF receptor induces the translocation of a constitutive complex between Grb2 and a guanine nucleotide exchange factor for Ras to the activated receptor. This translocation could link receptor activation to increased exchange factor activity by bringing the exchange factor to the subcellular compartment where its target, Ras, is located (that is, the plasma membrane), or by inducing a conformational change and/or tyrosine phosphorylation of the exchange factor.

In *C. elegans* the Grb2 homologue Sem-5 is implicated in the control of Ras signalling by the receptor tyrosine kinase Let-23, and a *Drosophila* homologue of Grb2 has recently been identified^{26,27}. The capacity of Grb2 to mediate the EGF-induced activation of a guanine-nucleotide-exchange factor for Ras may therefore represent an evolutionary conserved mechanism to relay receptor signals to Ras. □

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Imprinting makes its mark

Work published over the past month provides insight into the mechanism of genomic imprinting in mice and the possible phenotypic consequences of imprinting in humans.

THE phenomenon of imprinting — the preferential expression of the maternally or paternally inherited allele — is in itself fascinating. But as several studies^{1–6}, including two in the latest issue of *Nature Genetics*^{1,2}, now make abundantly clear, findings on imprinting will have considerable bearing on areas as diverse as gene expression, development, cancer and evolution. These new discoveries further our understanding in two ways: first, by extending part of what is known about murine imprinted genes to their homologous loci in humans; and second, by offering encouraging clues as to the precise molecular mechanisms responsible for the imprint itself.

Four genes in mice are known to be imprinted (see table), the most recently discovered being one that encodes a small nuclear ribonucleoprotein (*Snrpn*) whose human homologue may play a part in the aetiology of the imprinting disorder called Prader–Willi syndrome (reviewed in ref. 7). Much attention has also been paid to the insulin growth factor 2 (*Igf2*) and *H19* genes, which are a pair of physically linked⁸, reciprocally imprinted loci on mouse chromosome 7. In humans, these two genes map to chromosome 11p15, a region that is frequently inherited as two paternal copies in patients with Beckwith–Wiedemann syndrome (BWS), a fetal overgrowth disorder characterized by predisposition to tumours including Wilms' tumour⁹. It had been speculated that human *IGF2* is synonymous with the BWS gene, because of its effects on growth and its paternal-specific expression¹⁰. But there had been no direct evidence that any of the imprinted genes in mice were similarly modified in humans.

Writing in *Nature Genetics*, two groups show definitively that *IGF2* is indeed paternally expressed in human fetuses. Ohlsson *et al.*¹ and Giannouka-

kis *et al.*² examined polymorphisms in the coding sequence and the 3' untranslated region, respectively, of *IGF2* in a total of 19 fetal tissue samples. Six of these cases proved to be fully informative, allowing the investigators to distinguish between the parental alleles, and then show that only the paternally derived allele was being expressed.

IMPRINTED GENES IN THE MOUSE			
Gene	Expressed allele	Mouse chromosome	Human chromosome
<i>H19</i>	Maternal	7	11p15
Insulin growth factor 2 (<i>Igf2</i>)	Paternal	7	11p15
Insulin growth factor 2 receptor (<i>Igf2r</i>)	Maternal	17	6q25–q27
Small nuclear ribonucleoprotein N (<i>Snrpn</i>)	Paternal	7	15q12

Although in many respects this result was to be expected, it is important to clarify the imprinting status of this and other genes in humans, and indeed in other species apart from mouse, if sensible predictions about the evolutionary role of imprinting are to be made.

Given the position of *IGF2* at 11p15.5 and its possible involvement in BWS, Ohlsson *et al.* also examined the parental imprinting of *IGF2* in a perinatal BWS patient, and found that it was imprinted as well. But, as shown a fortnight ago in *Nature*^{3,4}, the imprinting of *IGF2* and also *H19* may be relaxed in tumours associated with BWS.

Rainier *et al.*³ and Ogawa *et al.*⁴ examined a total of 72 patients with Wilms' tumour, based on the association of the tumour with BWS and the known expression of *IGF2* and *H19* in the kidney. After discounting those cases caused by loss of one allele, and those in which the alleles could not be distinguished, they found that a total of 14 out of 19 Wilms' tumours exhibited expression of both *IGF2* alleles. In addition, Rainier *et al.* found that *H19* is expressed biallelically in two out of seven tumours. In one example, both genes could be demonstrated to be expressed biallelically, suggesting a possible disturbance in a hypothetical switch between the two genes which directs their contrasting allele-specific pattern of expression⁸. More generally, given the frequency of

the 'loss of imprinting', both groups propose that this relaxation may be a common contributory factor to the onset of carcinogenesis.

One mechanism that might explain the relaxation of imprinting would be an effect of methylation, which has been correlated with the repression of transcription of many genes. Indeed, work from Ferguson-Smith and colleagues⁵ on the mouse *H19* gene is consistent with this view. Using methylation-sensitive restriction enzymes, they found that methylation at specific sites within the *H19* promoter is confined to the repressed paternal allele. Moreover, this allele is less sensitive to nucleases, indicative of a more condensed and less-accessible chromatin structure. A similar pattern of methylation was seen by Stoger *et al.*⁶ at the 5' end of the non-expressed paternal allele of the murine insulin-like growth factor 2 receptor (*Igf2r*). But in this case, an even more striking methylation event occurs within a 5' intron of the expressed, maternal allele. Methylation at a CpG island in this intron was found only on the maternal allele. This is significant because unlike the promoter, which is methylated after fertilization, the intron is already methylated in the female gamete, consistent with the idea of an imprinting signal. So for *Igf2r*, the 'imprinted' allele and the expressed allele are one and the same — something of a surprise. Further studies of *Igf2r*, in particular in mice that lack the enzyme for DNA methylation, should help to resolve the tantalizing link between methylation and imprinting, which may have evolved from the host defence system employed by bacteria to destroy foreign DNA¹¹.

Kevin Davies

Kevin Davies is Editor of *Nature Genetics*.

Also in this month's *Nature Genetics*: a gene for familial glaucoma mapped to chromosome 1; determining chromosomal regions of identity by 'genomic mismatch scanning'; a role for angiotensinogen in pregnancy-induced hypertension; and a full report of the first international *Nature Genetics* conference in Washington, DC.

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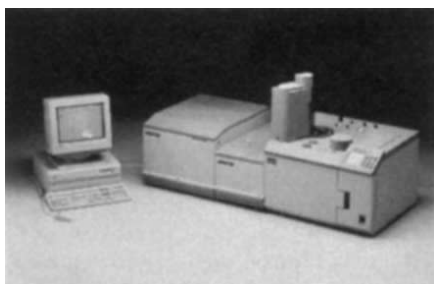
Modes of chromatography

Featured this week — antibody purification kits, a diode-array detector for liquid chromatography, software for chromatography applications, columns, media and a collection of catalogues for one-stop shopping.

E-Z-SEP is a **protein purification system** from Middlesex Sciences that consists of a mixture of polymers specially formulated for the purification of IgG and IgM monoclonal and IgG polyclonal antibodies with greater than 90 per cent purity (*Reader Service No. 100*). Using a non-charged polymer network system to entrap the protein of interest and partition out other proteins and lipids, E-Z-SEP has been designed to provide a useful alternative to current methods of column chromatography and membrane separation techniques. Specific formulations have been developed for preparing high-purity gamma globulins directly from clarified ascites fluid, cell culture supernatants and bioreactor media. The basic protocol yields all subclasses of gamma globulins, as well as all isoelectric point variants within a subclass. Middlesex Sciences says that purification requires less than 30 min of hands-on time and a total processing time of less than 3 h. In addition, the company says that E-Z-SEP does not cause denaturation of immunoglobulins, or raise the potential for contamination.

■ Gas chromatography

The new System 2000 **gas chromatography/Fourier transform infrared system** (GC/FR-IR) from Perkin-Elmer is designed to combine GC and FT-IR without compromising the performance of either (*Reader Service No. 101*). The instrument provides a



Perkin-Elmer's combined gas chromatography and Fourier transform infrared system.

spectrum of each pure component eluted from the gas chromatograph, allowing for the identification of each component and even distinguishing structural isomers of the same molecular mass. The system contains a low-volume (<100 μ l) light-pipe system that provides low-nanogram sensitivity. Scan rates up to 20 spectra(s) ensure no loss of resolution for capillary columns. Automatic baseline correction removes the effects of column bleed and atmospheric variations, enhancing both the speed and reliability of

the analysis, according to the company. The time-resolved infrared software provides three-dimensional or contour plotting. The system is available as a dedicated instrument, or as part of the multi-application System 2000, with up to three other FT-IR sampling stations.

Shimadzu Scientific introduces the QP-5000, a compact **gas chromatography/mass spectroscopy system** (GC/MS system) that is designed to offer increased sensitivity, mass range and ease of use (*Reader Service No. 102*). The QP-5000 operates in a mass range from 10 to 700 amu at a resolution of one mass unit or 2M (determined at a 50 per cent valley). The higher mass range allows the user to incorporate an optional direct inlet for qualitative analysis of less volatile samples, such as polymers. The QP-5000



Shimadzu's QP-5000 gas chromatography/mass spectroscopy system.

uses Shimadzu's new GC-17A with advanced flow control as the gas chromatograph, which allows the user to set easily column flow or linear velocity, split ratios or split flow, total flow or pressure, as well as programmed flow and pressure. Shimadzu says that the automated set-up increases the reliability of each reading. The GC-17A also automates the ignition of the FID flame. The QP-5000 is controlled by the Class-5000 software package, which operates in the Windows 3.1 environment on a personal computer. The software allows manual and automated quantitative and qualitative methods, including the ability to build private libraries or to use the National Institutes of Standards and Technology library. The system measures 710 $\times$ 510 $\times$ 550 mm, including the MS, GC and vacuum pumps.

■ LC/HPLC

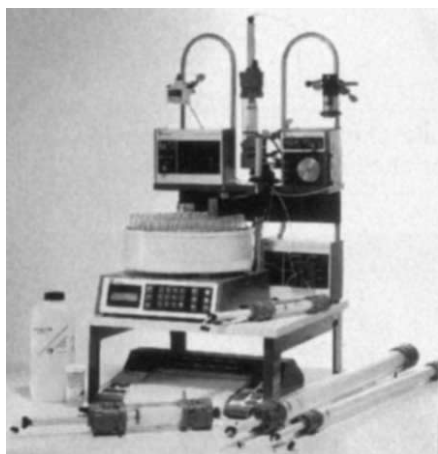
The HP 1050 series **diode-array detector for liquid chromatography** supplied by Hewlett-Packard is available as a stand-alone detection system or as part of an HP 1050 series high-performance liquid chromatography (HPLC) modular system



HP 1050 series diode-array detector for liquid chromatography is available as a stand-alone or as part of a modular system.

(*Reader Service No. 103*). For automated work, the detection system features a PC-based controller, an HPLC^{3D} ChemStation (DOS series) running under Microsoft Windows. The HP 1050 is designed for research analysis and for routine work using spectral libraries, which are used to compare the peak's spectra with that of a standard archived electronically, for positive confirmation of quantitative results. A typical high-performance system is priced under US\$25,000 and includes an HP Vectra 486/66U PC, a laserjet printer and the software (the detector is US\$13,430). The new detector acquires three-dimensional data continuously during elution of the peak. The third spectral dimension provides the chromatographer with more data per unit time for more reliable results. For example, Hewlett-Packard says that the HPLC^{3D} ChemStation's software can confirm the purity of a peak, while quantifying the response against calibration standards. Automated features of the HP series diode-array detector include peak qualification (based on absorbance ratios of two or more simultaneously acquired signal wavelengths), peak-purity calculations (based on comparisons of UV-visible absorbance spectra recorded during elution), positive peak identity (based on spectral comparisons with archived UV-visible absorbance spectra from spectral libraries), and self-validating optics and micro-processors (based on holmium oxide filter for wavelength calibration and electronic system verification feature to verify data transfer from detector to PC).

GradiFrac is a low-pressure, **preparative chromatography system** from Pharmacia for purifying proteins, peptides and other biomolecules (*Reader Service No. 104*). The system supports a wide range of applications, which require either isocratic or gradient elution. It is built around the GradiFrac, a



GradiFrac low-pressure preparative chromatography system.

new combined controller and fraction collector and includes a GradiFrac, a P1 pump, a UV1 monitor, a REC 102 chart recorder, a IV-7 injection valve, a mixer, two solenoid valves PSV-50 and a rack. This compact system, which takes up 0.22 m² of bench space, is portable and designed to be quick and easy to assemble with tubing connections that require no flanging. Pharmacia offers a complete line of media and columns for this system for a broad range of applications.

The new Series 1100 HPLC systems and DataControl PC/Windows software from Cecil Instruments have a modular design and can be configured as simple isocratic gradient systems, with or without PC data acquisition and manipulation, or as complete automatic gradient systems (*Reader Service*



Series 1100 HPLC systems and data control Windows-based software.

No. 105). A choice of UV detectors and other alternative detectors is available, allowing a wide range of applications to be addressed. The high-performance, dual-piston analytical pump head of the Series 1100 pump module can be easily interchanged with a range of alternative heads that include microbore, preparative and inert

(titanium) pump heads — greatly extending the applications of these modules and systems. The DataControl PC-based software operates within MS-Windows and offers full pump control for dual or triple pump gradient systems. Dual channel operation is available as standard and this can be expanded, with a suitable PC, for six-channel simultaneous operation. The advanced data processing and report generator available within the DataControl software enables the user to generate high-quality results and reports easily and reproducibly with all methods stored for rapid recall.

■ Software

GynkoSoft is a **chromatography data system** from Severn Analytical that integrates all the components of an HPLC laboratory (*Reader Service No. 106*). The system can be used to control chromatography instruments from manufacturers other than Severn Analytical and offers networking capability with other PCs or a central laboratory information management system. The algorithms for peak detection and integration are designed to give a precise baseline determination, allowing the user to optimize each peak individually and automatically. Data processing can be executed while data acquisition is in progress. Operation is intuitive, with a Windows-oriented interface, a help system and dialogue boxes to assist the user. Error messages and dialogue guides help the inexperienced user and cut down on learning time. Automated comparisons of spectra, peak purity test, peak identification by spectra, contour diagrams and three-dimensional plots can be implemented automatically during batch operation.

Galactic Industries has improved the capabilities of GRAMS/386, a Microsoft Windows-based application, with the addition of a **method editor for applications in chromatography** (*Reader Service No. 107*). The method editor is completely menu-driven and provides a logical organization of chromatographic functions. The software can work with a variety of chromatography data types such as liquid chromatography, gas chromatography (GC), high-performance liquid chromatography (HPLC), size exclusion chromatography/gel permeation chromatography, diode-array/HPLC, GC/mass spectroscopy and GC/FT-IR. The advanced graphic capabilities found in the method editor provide the chromatographer with data visualization screens for displaying information such as calibration curves or timed events. Reports and printouts are generated from within the GRAMS/386 environment. Calibration reports can be output to hardcopy and method files can be printed as they appear in the method editor. Method file printouts are organized sectionally by calibration, peak picking and run control. The method editor also allows changes/updates to method files. Bad

calibration samples can be removed with a click of the mouse, Galactic says. GRAMS/386 runs under Microsoft Windows versions 3.0 and higher on 386 and 486 PC and compatible computers. A maths co-processor and VGA display are required.

Five computer-based programs on **HPLC training and troubleshooting** are described in Savant's new four-page brochure: Introduction to HPLC, CLC-10; Method Development in HPLC, CLC-20; HPLC

Savant's HPLC training and troubleshooting software program.

Equipment, CLC-30; Troubleshooting HPLC, CLC-40; and Separation Modes of HPLC, CLC-50 (*Reader Service No. 108*). The first module, Introduction to HPLC provides the user with an overview of the technology and introduces separation principles, mechanisms, retention and instrumentation. The troubleshooting program includes an 'expert system' that identifies problems from symptoms and suggests solutions, allowing this module to be used both as a learning and diagnostic tool. All programs feature interactive and 'electronic chromatographs' that show results of changes on a synthesized chromatogram when experimental conditions are changed. Results are recorded automatically in an electronic notebook. Self-testing is provided with onscreen quizzes and 'hot words' provide instant onscreen definitions of chromatographic terms. The programs require an IBM PC or compatible computer with 286 or higher processor, at least 1 MB RAM, preferably more, a hard disk with 2.5 MB of free space per title, colour VGA adapter and monitor, Windows 3.0 or later and mouse.

■ Columns and media

Eka Nobel AB is introducing Kromasil 60 Å spherical high-performance **chromatography silica**, which because of its very

narrow pore-size distribution is designed to have a higher accessible surface area than competing 60 Å silica packings (*Reader Service No. 109*). The company says that the material has the same high mechanical strength and surface properties as Kromasil 100 Å. Kromasil 60 Å is available as plain silica in particle sizes of 5, 7, 10, 13 and 16 µm and in analytical to multi-kilogram quantities.

Phenomenex now offers a wide range of **packed columns in a metal-free/polyether-etherketone (PEEK) assembly** (*Reader Service No. 110*). The columns are designed to overcome many of the limitations of stainless steel components in a growing number of biotechnology and ion chroma-

drugs, vitamins, catecholamines, organic acids and peptides.

Integrated Separation Systems now offers its **Pentax preppacked ceramic hydroxyapatite columns** in two sizes, both of which



Pentax preppacked ceramic hydroxyapatite columns from ISS.

are biocompatible glass chromatography columns suitable for FPLC and HPLC use (*Reader Service No. 111*). Designed primarily for the purification of immunoglobulins and biologically-active enzymes, these columns will fit Pharmacia FPLC systems and can be adapted to HPLC systems from Waters, Rainin, Shimadzu and others. The smaller column is packed with 2 µm ceramic hydroxyapatite beads and has an operating pressure limit of 850 p.s.i.. The column is intended for analytical purposes and for methods development. The larger column is suitable for preparative scale separations and is packed with 10 µm ceramic hydroxyapatite beads with an operating pressure limit of 250 p.s.i.

The new **polymeric reversed phase PLRP-S 300 Å columns** from Polymer Laboratories are designed to replace conventional silica materials for peptide and protein analysis and purification (*Reader Service No. 112*). The homogeneous PLRP-S polystyrene/divinylbenzene surface has retention characteristics that are closely analogous to those of conventional silica alkyl-bonded packings, the company says. PLRP-S high-performance analytical and preparative columns, which offer long term mechanical and chemical stability, are available in narrow-bore, analytical, preparative and process-scale column dimensions, and as bulk media.

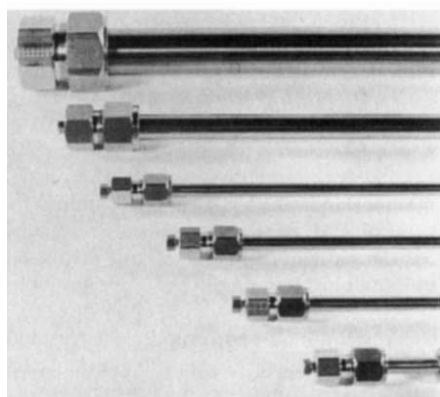
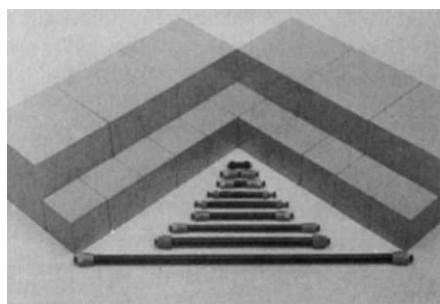
3M and Pierce recently introduced Emphase — **activated affinity chromatography beads** (*Reader Service No. 113*). This new biosupport technology is based on preactivated beads composed of hydrophilic, crosslinked bis-acrylamide/azlactone copolymer. The rigid bead architecture provides a higher surface area and pore volume than conventional supports, resulting in a higher binding capacity and higher flow rates, Pierce says. The new Emphase beads allow researchers to couple high yields of proteins in less than an hour. The 60-µm

diameter beads will withstand 100 p.s.i and provide flow-through rates of 3,000 cm h⁻¹. Pierce says that there is no leaving group when a ligand is coupled to the beads, resulting in safe chemistry and stable amide linkage. 3M Emphase beads are stored dry and are reusable (over 100 cycles with 99 per cent capacity has been demonstrated, the company says). A 1-g package of the beads will generate 8–10 ml of gel.

■ Ion chromatography

The use of **electrochemical detection (ELCD)** in HPLC and ion chromatography is a sensitive and time-tested method that can provide a complement to traditional detection methods (*Reader Service No. 114*). The method can be tailored to organic trace and ultratrace analysis. The Metrohm ELCD equipment now being offered by V. A. Howe, which consists of the Metrohm 641 electronic unit and the Metrohm 656 wet chemical unit, can be used to detect numerous oxidizable substances down to the picogram range with high selectivity, the company says. It has been designed so that it can be used in series with, or parallel to, traditional HPLC detectors. The detector can also be built into the Metrohm 690 ion chromatograph as a second detector in series. This, the company says, opens up additional possibilities, not least the determination of nitrite down to the sub-parts-per-billion level.

Millipore/Waters announces the introduction of a new technology — isomigration time mode — for the company's **capillary ion analyser** (*Reader Service No. 115*). Isomigration time mode is designed to ensure accurate and reliable peak identification, even when there are variations in analyte concentration levels between samples. Waters says that this new technology minimizes migration time shifts caused by variations in sample conductivity, eliminating the risk of peak misidentification. This makes capillary ion analysis an effective and dependable technique for ion analysis in



Metal-free/PEEK HPLC columns (top) and reverse-phase columns from Phenomenex.

tography applications. The columns meet the stringent requirements of mechanical strength, biocompatibility and chemical resistance that these applications demand. The company also offers a complete line of biocompatible parts and accessories for HPLC systems, such as tubing, connectors, valves, solvent inlet and in-line filters. In addition, Phenomenex is offering silica- and polymer-based reversed phase chromatography columns in a variety of pore diameters and bonded-phase materials. Monomeric and polymeric reversed phase chromatography supports confer different selectivities, depending on the physical properties of the solute, while ligand chain length determines hydrophobicity. The company says that its reversed phase chromatography columns offer improved resolution of small molecules such as



Waters capillary ion analyser now comes with isomigration time mode technology.

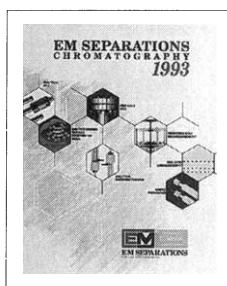
the research and development, quality control and process control laboratory where reproducibility is essential. Additional features of the Waters capillary ion analyser include: the ability to monitor wavelengths as low as 185 nm, detection by direct or indirect ultraviolet for analysing anions, cations, organic acids, surfactants and transition metals, and temperature control of the separation compartment from sub-ambient to 45 °C.

■ Catalogues

The 1993 EM Separations **chromatography catalogue** includes a complete listing of

products for analytical and preparative HPLC, high-performance biochromatography, HPTLC and sample preparation (*Reader Service No. 116*). The new catalogue features Aluspher, a new spherical alumina support that can be used with high pH mobile phases, a new chiral bonded B-cyclodextrin phase, and the E. Merck LiChroprep direct scale-up system that is designed to provide reproducibility from analytical to process scale. Also included are E. Merck LiChroCART cartridge columns. Tentacle ion exchange and tentacle affinity bioseparation media are available in bulk quantities and in prepacked cartridges.

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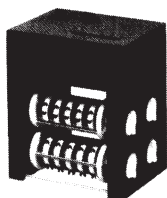
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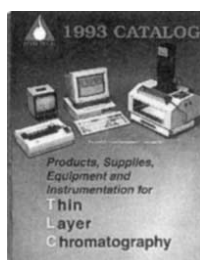


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The 1993 Analtech **thin layer chromatography catalogue** is now available and



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features a complete line of TLC plates, accessories and instrumentation (*Reader Service No. 117*). The company's TLC Uniplates are made in a variety of adsorbents, including hard layer and soft layer silica gel, HPTLC, reversed phase, alumina and cellulose. Uniplate formats include preadsorbent spotting areas, channelled adsorbent and scored 'snap apart' TLC Uniplates. The catalogue also features the Uniscan video densitometer and a full line of TLC accessories, including new sample application devices, UV lamps, transilluminators, viewing cabinets and photodocumentation systems.

The new J.T. Baker 1993-1994 **laboratory reagents and chromatography products catalogue** contains 400 pages of

specifications, applications and references for some 4,000 listings (*Reader Service No. 118*). More than 300 new products are featured, including ultrapure bioreagents, returnable high-purity solvent containers, atomic spectral standards and Empore extraction disks for Environmental Protection Agency methods, and a new generation of solvent purity for analysis by gas chromatography.



J.T. Baker

The new 250-page TosoHaas **catalogue of chromatographic columns and media** products features more than 500 products for the biopharmaceutical researcher involved in analysing, isolating and purifying proteins, peptides, enzymes, nucleic acids or other small biomolecules (*Reader Service No. 119*). The introduction of the rigid size exclusion chromatography materials has greatly improved the performance of this technique for the separation of biomolecules and organic polymers, and a special section dedicated to size exclusion chromatography and containing technical data and information on applications can be found in the catalogue. TosoHaas offers a complete line of porous silica- and polymer-based size exclusion chromatography materials. The catalogue also highlights products for modes of chromatography including ion exchange, hydrophobic interaction, reversed phase and affinity, as well as prechromatographic applications, specialty chromatography columns, accessories and standards.

Over 2,000 products are featured in the 1993 Sigma-Aldrich **chromatography catalogue** (*Reader Service No. 120*). For the first time, Sigma, Aldrich and Fluka's chromatography-related products are assembled in one catalogue and combined with many new products, including Flukabrand ion pair reagents, SigmaChrom high-performance affinity columns, capillary gel chromatography columns, HPLC columns, thin layer chromatography plates and gels, flash chromatography columns, Sigma-Aldrich HPLC solvents, environmental standards, Vydac HPLC columns for protein and peptide separations, Reacta-Sil derivatization reagents, and media for column chromatography.

Amicon's 64-page colour 1993 product catalogue presents over 450 products for **ultrafiltration, chromatography and membrane affinity chromatography** (*Reader Service No. 121*). In addition to product specifications, ordering information and prices, the catalogue's new applications guide provides information about the company's membrane filtration products for the laboratory. For larger-scale applications, reference to spiral-wound and hollow-fibre cartridges and systems is included. Also included in Amicon's catalogue are laboratory columns for liquid chromatography and a wide range of chromatography media with various choices for the purification of proteins, enzymes and peptides. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.